

Otitis Media with Effusion: Key Factors

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Abstract

Otitis media with effusion (OME) is a disease characterised by inflammation of the middle ear and changes in middle ear mucosa from a columnar to a more secretory type epithelium, with a proliferation of goblet cells and mucus glands. There is excessive production of mucus, resulting in the accumulation of a viscous effusion in the middle ear cleft. Various factors have been implicated in the aetiology of the disease including bacteria, Eustachian tube dysfunction, allergy and craniofacial abnormalities. It has been suggested that laryngopharyngeal reflux could be an inflammatory co-factor and possible cause of many upper respiratory disorders including OME.

The aims of this thesis were to examine effusions for the presence of gastric juice and were also concerned with the biochemical and rheological characterisation of effusions.

Acidic proteinase (pepsin) activity measured at pH2.2 using the N-terminal assay was detected in 29% of effusions and 91% of these samples contained pepsin/pepsinogen protein at elevated levels compared to serum (using an ELISA). As other serum protein levels in effusions were of the same order as serum reference levels, the source of the activity was unlikely to be from a transudate of plasma and rather due to the reflux of gastric contents into the middle ear. The data suggests that reflux may be a primary factor in the initiation of OME in children.

Middle ear effusions are mucin-rich secretions that demonstrate a lack of degradation on storage. They contained at least two distinct mucin populations, MUC5B is the major mucin and MUC5AC is present at much lower levels. MUC5B had a significant correlation with effusion specific viscosity suggesting that it is responsible for the rheological properties of the effusion.

Interleukin-8 (IL-8) levels in effusions had significant correlation with MUC5AC and it may be that MUC5AC is produced in response to IL-8 in the middle ear during the inflammatory process of glue ear. Neither IL-6 nor IL-8 levels correlated with MUC5B content. It is likely that a different stimulus or other cytokine is responsible for the regulation of MUC5B. There was a significant correlation between IL-6 and IL-8 levels in effusions demonstrating that one cytokine could stimulate the secretion of the other.

Further studies developing from the work presented in this thesis would involve analysis of effusions for other components of gastric juice, such as gastric lipase and intrinsic factor, to

confirm the role of reflux in OME. A study following children from the diagnosis stage through the disease course and grommet insertion for glue ear with awareness of signs and symptoms of reflux would assess the proportion of children with glue ear associated with reflux. An animal model for reflux could be set up, instilling gastric juice components into the middle ear via the nasopharynx/ET to see if an effusion develops. Effusions could be analysed for other cytokines (such as IL-1 β or TNF- α) to see if levels correlate with MUC5B content.

Publications and reviewed abstracts

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Abbreviations

AOM	acute otitis media
Asp	aspartate
BSA	bovine serum albumin
C	Celsius
cDNA	complementary DNA
CsCl	caesium chloride
Da	Daltons
DAB	3'3-diamino tetrahydrochloride
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
ET	Eustachian tube
g	centrifugal force
GOR	gastro-oesophageal reflux
GORD	gastro-oesophageal reflux disease
GuHCl	guanidine hydrochloride
H ₂ O ₂	hydrogen peroxide
HCl	hydrochloric acid
Hz	Hertz
IFN	interferon
IL	interleukin
kb	kilobase
LOS	lower oesophageal sphincter
LPR	laryngopharyngeal reflux
M	molar
MG1	high molecular weight salivary mucin
MG2	low molecular weight salivary mucin
M _r	molecular weight
mRNA	messenger ribonucleic acid
MUC	mucin gene

n	number
NaCl	sodium chloride
NaN ₃	sodium azide
NDS	non-dialysable solids
OD	optical density
OME	otitis media with effusion
P	probability
PAGE	polyacrylamide gel electrophoresis
PAS	periodic acid Schiffs
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethyl-sulphonylfluoride
R ²	goodness of fit of non linear regression
r ²	goodness of fit of linear regression
RNA	ribonucleic acid
rpm	revolutions per minute
s	second
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
TNBS	trinitrobenzene sulphonic acid
TNF	tumour necrosis factor
TVP	tensor veli palatini
UOS	upper oesophageal sphincter
v/v	volume for volume
VNTR	variable number tandem repeat
V _O	void (excluded) volume
V _T	total volume
vWF	prepro-von Willebrand factor
w/v	weight for volume
w/w	weight for weight
η _{rel}	relative viscosity
η _{sp}	specific viscosity

CHAPTER 1

INTRODUCTION

Chapter 1 Introduction

The middle ear constantly secretes mucus, which is transported via cilia from the middle ear through the Eustachian tube toward the nasopharyngeal orifice (Ohashi and Nakai, 1991) where it can be swallowed (Figure 1.1). The mucociliary transport system defends the middle ear against inhaled or invading particles including dust, irritant gases, bacteria, viruses and allergens. It protects the host by trapping and sweeping away these particles, preventing attachment to the respiratory epithelium and is thought to play an essential role in evacuating the middle ear pathogens that ascend along the Eustachian tube (Lin *et al.*, 2001).

1.1 Otitis media

Otitis media is an inflammation of the middle ear without reference to aetiology or pathogenesis. There are two subtypes: acute otitis media and otitis media with effusion. The rapid onset of signs and symptoms of inflammation in the middle ear is characteristic of acute otitis media (AOM). One or more local or systemic signs are present, otalgia, otorrhea, fever, the recent onset of irritability, vomiting or diarrhoea. The tympanic membrane is bulging and is opaque with limited or no mobility, indicative of a middle ear effusion. There may be discharge through a perforation of the tympanic membrane. By seven days in most cases the effusion has resolved and the individual is asymptomatic (Faden *et al.*, 1998).

Otitis media with effusion (OME) is a chronic inflammation of the middle ear in which an effusion is present but signs and symptoms of acute infection such as otalgia and fever are absent, and the tympanic membrane remains intact (Bluestone, 1999), which is

the most important distinction between OME and AOM. Hearing loss however is usually present in both conditions.

Otitis media with effusion can be graded by the duration of the disease, for example, acute (less than three weeks), subacute (three weeks to three months) or chronic (longer than three months) (Cheng and Young, 1997). A different system subdivides otitis media (including AOM) by the different histological and clinical characteristics observed, such as purulent (acute) (POM), serous (SOM), mucoid (MOM) and chronic (COM) (Agius *et al.*, 1995). Serous and mucoid otitis media frequently develop from Eustachian tube dysfunction and a serous or mucoid effusion develops in the middle ear cleft. In chronic otitis media, irreversible tissue pathology is observed and COM is frequently associated with increased numbers of mast cells in the middle ear cleft tissues (Hurst *et al.*, 1999). An overlap between the different types of otitis media is typical and intermediary types are also present as the definitions are not fully inclusive.

The research described in this thesis is based on studies on middle ear effusions (mucoid and serous) from children diagnosed with chronic otitis media with effusion where the effusion has been present for three months or more, therefore acute otitis media and acute and subacute OME will not be discussed further.

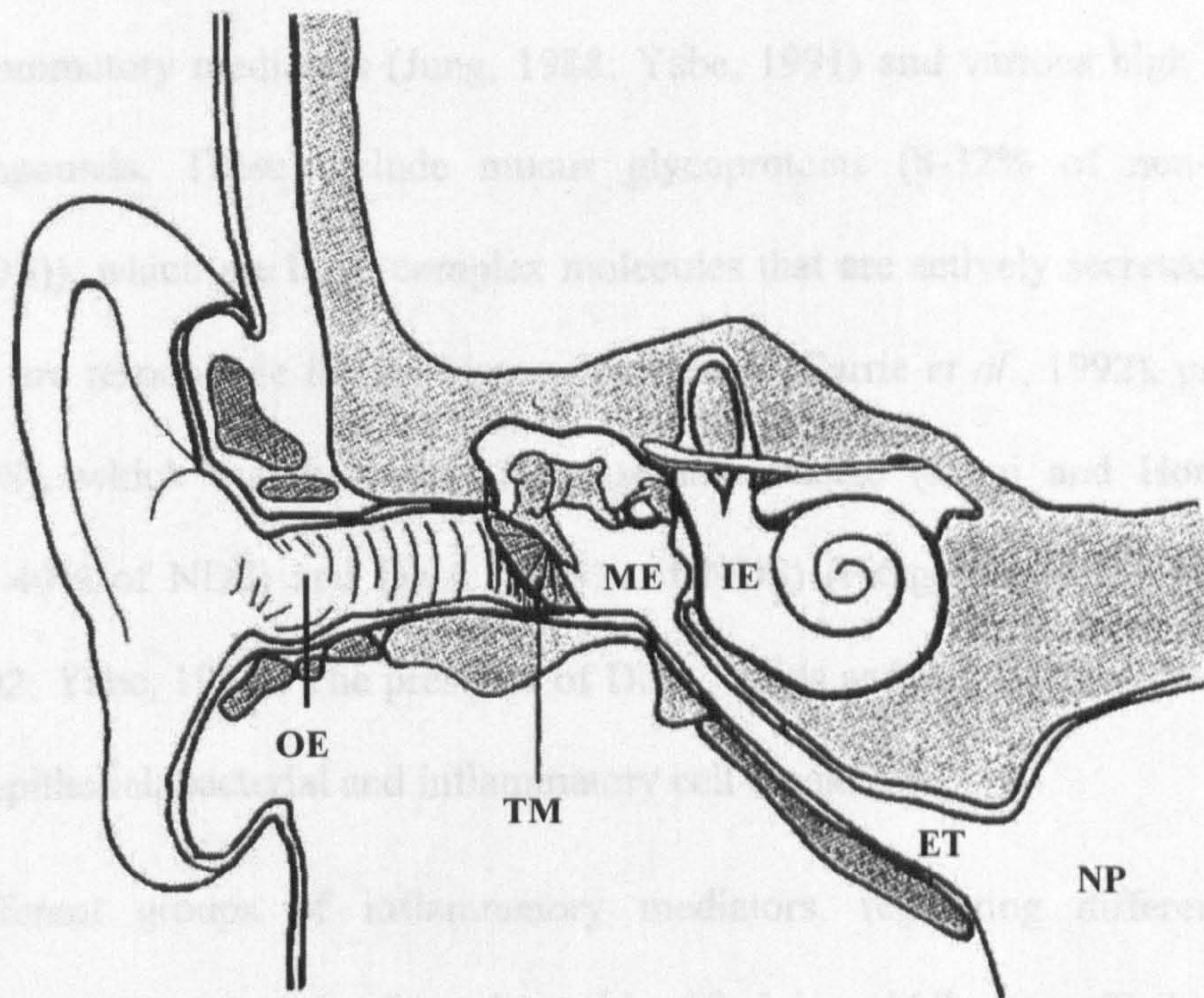
1.1.1 Development of OME

Otitis media with effusion (OME) is the most common cause of deafness in children in the developed world (Hunter *et al.*, 1994). The disease is characterised by inflammation of the middle ear and changes in middle ear mucosa from a columnar into a secretory type epithelium, with a proliferation of goblet cells and mucus glands (Sade, 1966; Lim *et al.*, 1973). This leads to excessive production of mucus and the accumulation of an

effusion in the middle ear cleft that cannot be cleared by the normal mucociliary transport mechanisms.

The presence of this effusion in the middle ear dampens ossicular mobility and impedes sound transmission, causing hearing losses of 15-50dB in the affected ear (Karver, 1998). When the effusion resolves, hearing usually returns to normal (Bluestone, 1999).

Figure 1.1 **Anatomy of the ear** (Straetmans *et al.*, 2001)



Outer ear (OE), middle ear (ME), inner ear (IE), tympanic membrane (TM), Eustachian tube (ET) and nasopharynx (NP).

1.1.2 Effusion composition

Effusions are composed of water, cells and cell debris, bacteria, electrolytes, inflammatory mediators (Jung, 1988; Yabe, 1991) and various high molecular weight compounds. These include mucus glycoproteins (8-32% of non-dialysable solids (NDS)), which are large complex molecules that are actively secreted into the effusion and are responsible for the viscous properties (Carrie *et al.*, 1992), protein (12-16% of NDS), which mainly comes from serum leakage (Mogi and Honjo, 1972), lipids (37-40% of NDS) and DNA (2-15% of NDS) (Fitzgerald *et al.*, 1987; Carrie *et al.*, 1992; Yabe, 1994). The presence of DNA, lipids and some protein would be as a result of epithelial, bacterial and inflammatory cell breakdown.

Different groups of inflammatory mediators, regulating different stages of the inflammatory response have been identified in middle ear effusions. Cytokines are potent mediators of inflammation and regulators of the immune response, produced by macrophages, neutrophils, lymphocytes and fibroblasts in response to stimuli such as endotoxin and viruses. Tumour necrosis factor-alpha (TNF α), interferon-gamma (IFN γ), interleukin-1-beta (IL-1 β), interleukin-2 (IL-2), interleukin-6 (IL-6) and interleukin-8 (IL-8) have all been identified in middle ear effusions (Yellon *et al.*, 1991; Yellon *et al.*, 1992; Hotomi *et al.*, 1994; Johnson *et al.*, 1997). Retention of cytokines in the middle ear cleft during OME may result in an ongoing inflammatory state that has the potential for mucosal changes, fibrosis, bone erosion and hearing loss. It may be possible that the cytokines remain in the middle ear from a previous inflammatory event, protected by the effusion from being metabolised.

There is a large variation in the appearance and nature of middle ear effusions. They vary from a clear effusion without distinct signs of infection to a cloudy or purulent fluid (Sade, 1979). There is also a variation in the consistency of effusions, from watery (serous) with a low viscosity to gel-like (mucoid) with a high viscosity. Effusions are usually classified on the basis of clinical inspection and their ability to flow on inversion (Carrie *et al.*, 1992).

1.1.3 Aetiology of OME

1.1.3.1 *Risk factors*

Various social factors have been implicated in the aetiology of OME, most of which are related to socio-economic status (such as poverty, poor nutrition or a large family) and all of which are probably mediated by an increased susceptibility to infection. A decreased socio-economic status increases the importance of environmental factors, which contribute to the risk of developing OME (Castagno and Lavinsky, 2002).

A number of risk factors are related to the home environment:

1. Presence of siblings in the home leads to an increased incidence of OME (Sassen *et al.*, 1997). This risk probably relates to exposure to potential middle ear pathogens and respiratory viruses harboured in siblings.
2. Breast-feeding exerts a protective effect against the development of OME. The mechanism of protection is likely to be the effect of maternal antibodies on middle ear pathogens (Owen *et al.*, 1993; Duffy *et al.*, 1997).
3. Exposure to tobacco smoke is a risk factor that impacts on the development and persistence of otitis media, especially early in life (Kraemer *et al.*, 1983; Owen *et al.*, 1993).

Children who attend day-care centres have a 2-3 fold increased risk of OME (Owen *et al.*, 1993; Nafstad *et al.*, 1999). Day-care plays a pivotal role in the spread of respiratory pathogens, bacterial and viral, to young and immunologically naïve children who can quickly become colonised with middle ear pathogens (Anainsson *et al.*, 1992).

1.1.3.2 Bacteria

Bacteria, especially *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis* have been cultured from up to 40% of middle ear effusions from children with OME (Giebink, 1989; Bluestone *et al.*, 1992; Liederman *et al.*, 1998; Rayner *et al.*, 1998; Kalcioglu *et al.*, 2002). The remainder of effusions are considered to be sterile by culture. Polymerase chain reaction (PCR)-based detection systems were shown to detect bacterial DNA in up to 80% of culturally sterile effusions (Post *et al.*, 1995; Post *et al.*, 1996; Liederman *et al.*, 1998) and a new culture-negative bacterium found solely in patients with OME was identified and named *Alloiococcus otitidis* (Faden and Dryja, 1989). This bacterium was present in up to 46% of effusions (Hendolin *et al.*, 1999). To further prove the validity of the PCR results in the detection of viable bacteria, workers have demonstrated the presence of bacterial messenger RNA (mRNA) in culture negative middle ear effusions (Rayner *et al.*, 1998). As mRNA has a half-life of seconds and can only be produced by viable microorganisms, this was considered further evidence of live pathogenic bacteria present in effusions.

In a chinchilla model it was demonstrated that free DNA and DNA from dead bacteria was cleared from the middle ear after three days (Aul *et al.*, 1998) showing that DNA from viable bacteria alone was detected by PCR. The animal model represents an acute infective effusion and the rapid degradation of free DNA in this environment does not necessarily mean that the same process will occur in a middle ear effusion from an individual with OME, a disease involving chronic inflammation. It has been shown by viscosity studies that human middle ear effusion homogenates incubated with DNA and DNase 1 inhibit the breakdown of DNA in a concentration-dependent manner by their ability to inhibit nuclease activity (Piezhong *et al.*, 2000) and that the reported presence

of non-culturable bacteria based on DNA (and RNA) detection by PCR (Post *et al.*, 1996; Rayner *et al.*, 1998) could still represent fossilized remains and not viable bacteria.

It has been suggested that the culture-negativity of effusions was due to the inability of culture to detect the active pathogen rather than a lack of pathogens and that bacteria were existing as a biofilm in a sessile state rather than a motile 'planktonic' state (Rayner *et al.*, 1998). Biofilms are known to be common in natural environments, on industrial equipment and increasingly in the human body, both on implanted medical devices and on certain tissue surfaces. In the biofilm state the bacteria are resistant to environmental stresses and are impervious to antibiotic treatment due to a decreased rate of replication and existence in a slow-growing or starved state. The antimicrobial agent may fail to penetrate the full depth of the biofilm (Costerton *et al.*, 1999; Wimpenny *et al.*, 2000). They are also resistant to the host's humoral defence system as there is a lack of accessibility to the bacteria by immunoglobulins and complement (Costerton, 1999), however they may still elicit an immune response resulting in the production of an effusion.

In a biofilm model of otitis media (Post, 2001), bacterial biofilms were clearly visible (using scanning electron microscopy) on middle ear mucosal samples of chinchillas with experimental otitis media induced by injection of non-typeable *H. influenzae* compared to the absence of a biofilm in control animals. From a time series experiment, an increasing density of bacteria and development of a matrix-encased biofilm was observed as time progressed. This study provides direct evidence for biofilm formation demonstrated using *H. influenzae* in this chinchilla model however there is no evidence for biofilm formation within the middle ear in humans. Several authors (Biedlingmaier

et al., 1998; Saidi *et al.*, 1999; Post, 2001) demonstrate biofilm formation on the grommet (the ventilating tube surgically placed through the tympanic membrane to restore aeration to the middle ear space (section 1.1.4) *in vitro* and *in vivo*. Bacterial attachment to this type of implant can become a source of chronic infection as they can rarely be eradicated without removal of the grommet and it is possible that planktonic cells periodically released from the bacterial biofilm could restart acute infection.

1.1.3.3 Viruses

Studies have indicated that viruses may play a role in the pathogenesis of OME (Arola *et al.*, 1990; Shaw *et al.*, 1995). Respiratory syncytial virus, rhinovirus, adenovirus and coronavirus nucleic acid have all been identified in effusions (Shaw *et al.*, 1995; Pitkaranta *et al.*, 1998; Moyse *et al.*, 2000). Direct evidence of co-infection by virus and bacteria in 11% of effusions was demonstrated in one study, supporting the concept that respiratory viruses, either alone or in combination with bacteria, are commonly associated with OME (Pitkaranta *et al.*, 1998). It is uncertain however whether the detection of viral nucleic acid represents persistent infection, residual non-infectious RNA or intercurrent infections. Viral upper respiratory tract infections may promote secondary bacterial infections by altering bacterial adherence, modulating host immune and inflammatory responses and impairing Eustachian tube function (Fireman, 1997). OME exhibits a bimodal distribution with peaks in spring and autumn, this is consistent with the rise in respiratory viruses or inhaled allergens demonstrating their possible role in the aetiology of the disease (Faden *et al.*, 1998).

1.1.3.4 Allergy

Allergy is considered to be a possible risk factor in OME. Allergic rhinitis has been identified in 42 % of children with OME (Mogi *et al.*, 1992) and there appears to be a higher incidence of chronic OME in children with allergy as compared with children without any evidence of allergy. This may be just an accidental association as there is no direct conclusive evidence that upper respiratory tract allergy induces OME (Fireman, 1988) but it may delay the resolution of the effusion by affecting Eustachian tube function.

1.1.3.5 Eustachian tube dysfunction

Eustachian tube dysfunction is thought to be involved in the development of OME. The Eustachian tube (ET) links the nasopharynx and the middle ear cavity. It is made of one-third bone (at the tympanic orifice) and two-thirds cartilage (at the pharyngeal orifice) and ciliated columnar epithelia predominates. The ET is essential in maintaining a healthy, well-aerated middle ear cavity. It ventilates the middle ear, protects it from pathogenic organisms in the nasopharynx via the mucociliary transport system, equilibrates pressure across the tympanic membrane and allows clearance of secretions from the middle ear into the nasopharynx. The tube opens intermittently to maintain ambient pressures in the middle ear, this occurs during swallowing, yawning or sneezing (Pinckney and Currarino, 1980).

In infants, the ET length is between 13 and 18mm compared to between 31 and 38mm by the age of seven years. The tube is at an angle of approximately 10° to the horizontal from the tympanic orifice to the pharyngeal orifice in infants compared to 45° in adults (Proctor, 1967). This could lead to possible clearance problems in children, but more

importantly it is possible that the angle of the tube in infants could have an effect on the function of the active opening mechanism of the ET (Ovesen and Borglum, 1998). Contraction of the tensor veli palatini (TVP) muscle opens the narrowest portion of the ET lumen to ventilate the middle ear (Sudo *et al.*, 1997). It has been demonstrated using a three-dimensional computer graphics reconstructive technique that in conjunction with the change in size and shape of the ET cartilage with age, the distance from the TVP muscle to the ET lumen is greater in children than in adults. A superiorly orientated rotation of the TVP muscle relative to the other components of the ET occurs with age (Sadler-Kimes *et al.*, 1989) and this angulation in adults may establish a mechanical efficiency for opening the tube that is absent in infants and young children. This may account for the opening or closing failure of the ET in infants and young children leading to obstruction or abnormal patency.

Obstruction of the ET can cause middle ear negative pressure, retraction of the tympanic membrane and hearing loss. This environment may promote the growth of bacteria and viruses already present in the middle ear and lead to inflammation and ultimately the development of a middle ear effusion. Abnormal patency of the ET results in the tube being open even at rest, which usually permits gas to flow readily from the nasopharynx into the middle ear. This effectively regulates middle ear pressure, however unwanted secretions from the nasopharynx, i.e. reflux or bacteria, can more readily gain access to the middle ear when the tube is abnormally patent.

Another explanation for the ineffective active opening of the tube may be related to the cartilage support. The amount and stiffness of the cartilage support in the ET are markedly less in infants than in older children and adults (Swarts and Rood, 1993) and the tubal cartilage is more compliant, therefore there may also be opening failure of the

ET due to its persistent collapse and the lumen may not open in response to contraction of the TVP muscle, resulting in a functional obstruction.

The constricted shape of the ET lumen in children (which is of a narrow, tubular configuration) means that it can be easily occluded with serous or mucoid effusions and may also prove to be a barrier to effective clearance of bacteria from the middle ear cavity (Suzuki *et al.*, 1998). Intrinsic mechanical obstruction of the ET can occur due to swelling, oedema or a lack of mucosal surfactants (Ovesen and Borglum, 1998) either within the ET or at its orifice in the nasopharynx and this can compromise ET function. Extrinsic obstruction can be due to the pressure of enlarged adenoids and in one study adenoidectomy and insertion of grommets was shown to improve the resolution rate (measured by time with recurrent effusion) of OME by 18% compared to the resolution rate with grommets alone (Gates *et al.*, 1987). At the end of the study however, the proportion of children remaining free of recurrence was the same in the two groups and therefore the role of adenoidectomy in the treatment of OME remains controversial. If adenoidectomy does improve the resolution rate this may be due to the removal of a physical obstruction of the ET or a source of ascending infection (Suzuki *et al.*, 1999).

An early theory to explain the mechanism of ET dysfunction in OME was that obstruction of the ET orifice in the nasopharynx would lead to a negative pressure in the middle ear as oxygen is absorbed and not replaced. As a result of the negative pressure, a transudate of fluid from the blood vessels would fill the ear causing an effusion. This is the 'hydrops ex vacuo' theory. The biochemical composition of the effusion found in OME is that of a usual inflammatory exudate, containing high molecular weight substances, and not just of a transudate of serum (Sade and Ar, 1997) and the 'hydrops

ex vacuo' theory does not explain the inflammation and metaplasia seen in the affected ear (Sade, 1966).

There is morphological increase in the dimensions of the ET lumen with age when it becomes larger and more like the shape of two cones connected by the narrow ends. This leads to an increased ability to clear secretions actively from the middle ear and to ventilate the middle ear more effectively.

1.1.3.6 Ciliary function

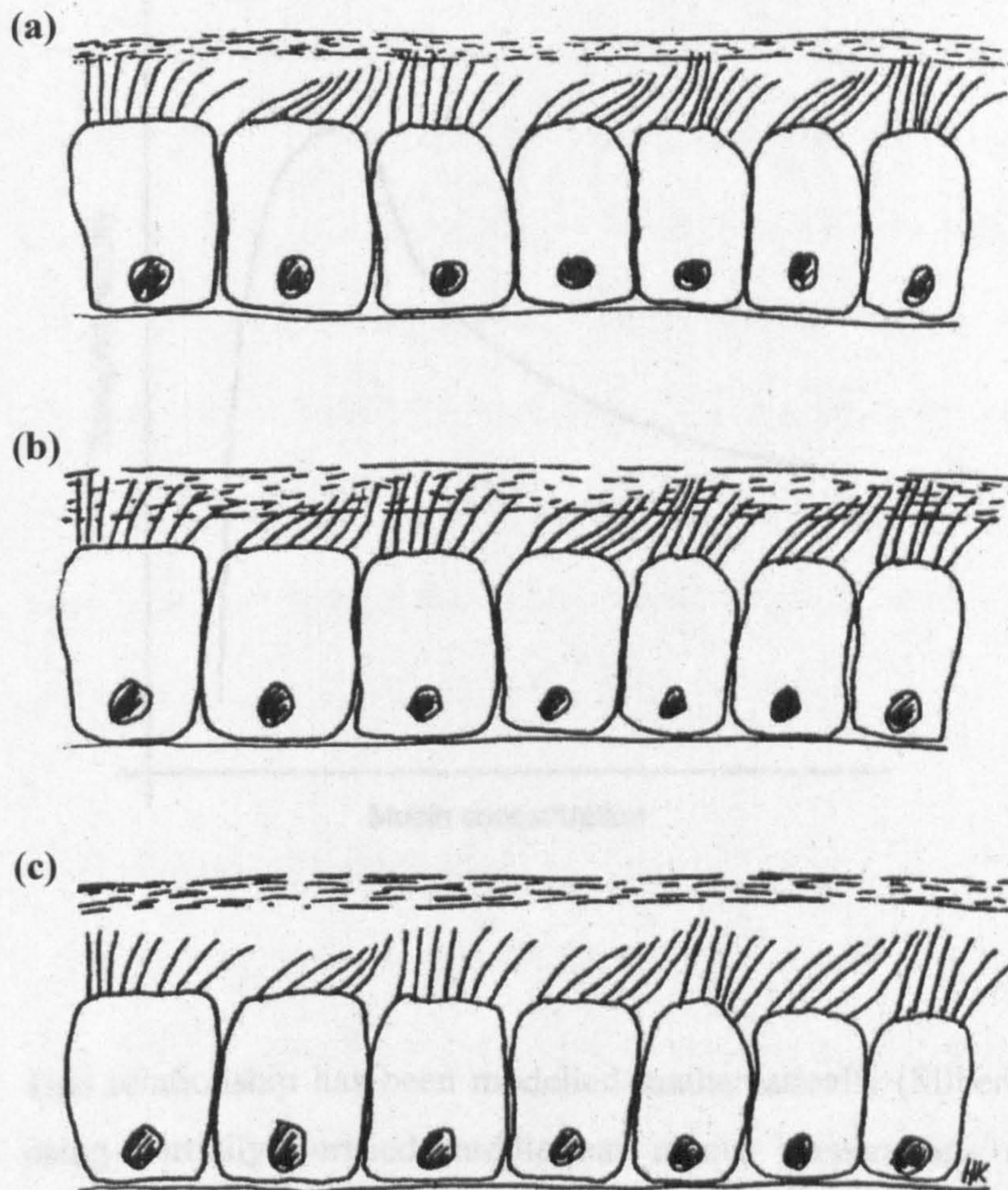
The middle ear and the ET contain ciliated epithelia (Houtmeyers *et al.*, 1999). The first evidence of an active mucociliary transport system in the middle ear was demonstrated in 1967 (Sade, 1967). Ciliated cells and goblet cells are abundant in the middle ear in the area near the ET orifice and their density becomes lower distal to the tube (Shimada and Lim, 1972). The ET is a pseudostratified columnar ciliated epithelium composed of ciliated cells, goblet cells and basal cells (Ohashi and Nakai, 1991). Cilia beat at around 10Hz within a layer (7-10µm) of low viscosity periciliary fluid upon which lies the more viscous mucus layer (0.5-2µm) (Quraishi *et al.*, 1998). The periciliary fluid layer is at a depth just less than the height of a fully extended cilium (Fig. 1.2). The tips of the cilia engage the overlying mucus layer and propel it towards the nasopharynx.

In OME there is a decrease in the number of ciliated cells and an increase in the number of secretory cells (Inagaki *et al.*, 1988) and it is likely that in this situation the system would fail to transport the mucus layer, thus impairing the clearance of the effusion. Reduced mucociliary function may be an important factor in the development of OME and the disease is present in approximately 84% of patients with primary ciliary dyskinesia (Jahrsdoerfer *et al.*, 1979; Pedersen and Mygind, 1982; Mygind *et al.*, 1983;

Greenstone *et al.*, 1985). Studies have shown that endotoxin is involved in the clinical development of OME and that the dysfunction of cilia caused by endotoxin may be responsible. Reduced ciliary activity was observed in the guinea pig middle ear in a dose-response manner, one and three days after inoculation with endotoxin from *Klebsiella pneumoniae*, measured by a direct and quantitative photoelectric method (Ohashi *et al.*, 1989).

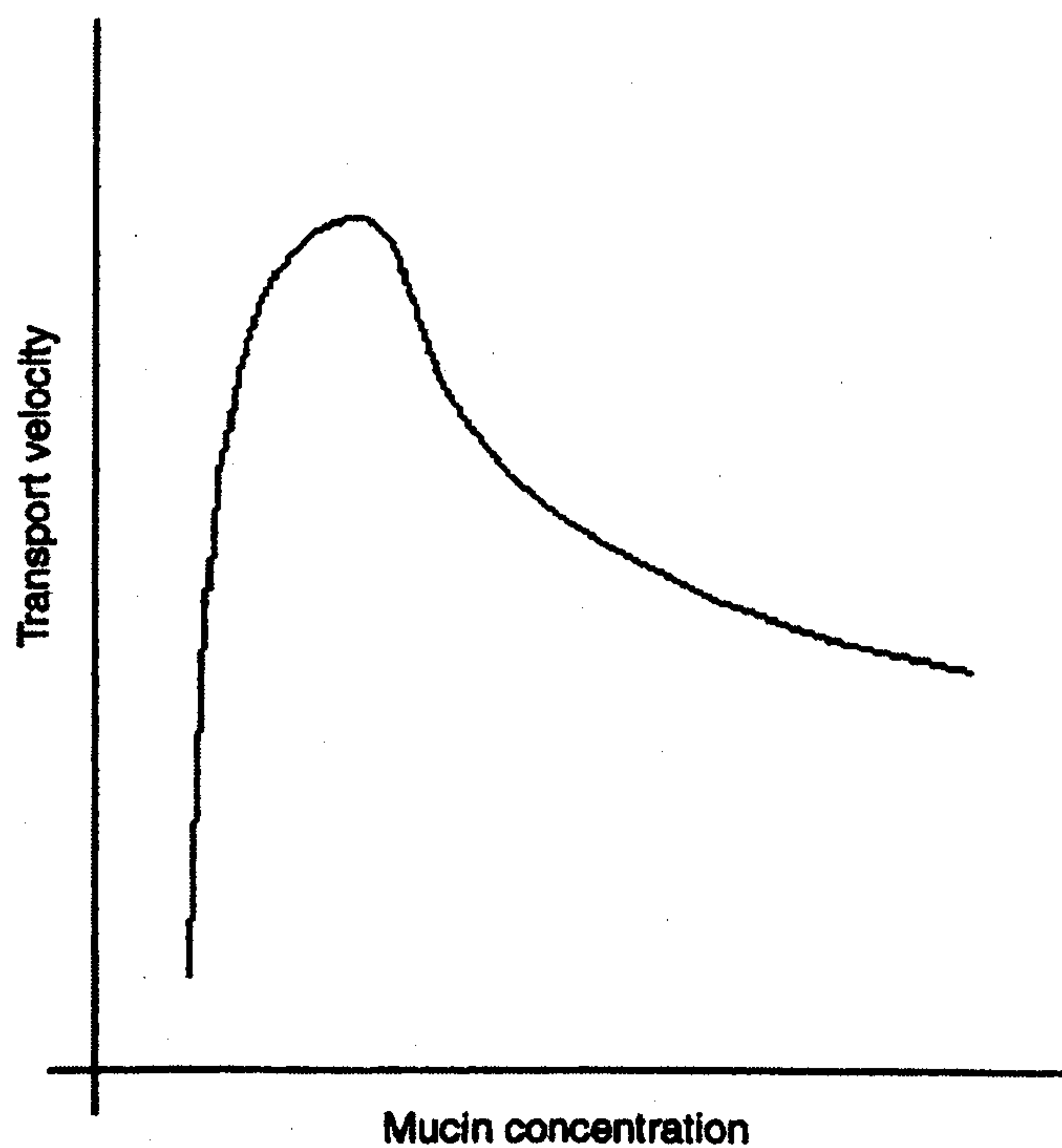
The mucociliary clearance of mucus is affected by its viscosity as the mucus has to be able to support a load and at the same time not distort the tips of the cilia in order to be cleared successfully. As mucus becomes more viscous there is a tendency for the ciliary beat frequency to decrease (Quraishi *et al.*, 1998). If mucus is too thick it is difficult for the cilia to deform it enough for it to move and if it is too thin the cilia cannot impart enough energy to it to transport it. There is an optimum viscosity at which mucus is effectively transported (Fig. 1.3) (Silberberg, 1983) and the viscosity of the mucus is determined by the nature and concentration of the mucins.

Figure 1.2 The effect of the depth of the periciliary layer on mucociliary clearance (Kubba *et al.*, 2000)



- (a) The ideal situation, where the tips of the cilia just penetrate the gel layer on their forward strokes, propelling it over the epithelium.
- (b) The periciliary layer is too shallow, so that the cilia are coupled to the gel layer throughout the forward and backward strokes, with no net movement over the epithelium.
- (c) The periciliary layer is too deep, so that the cilia are unable to impart enough energy to it to produce any movement of the gel layer.

Figure 1.3 The relationship between mucus concentration (viscosity) and mucociliary clearance rate



This relationship has been modelled mathematically (Silberberg, 1983) and confirmed using partially purified middle ear mucus preparations at varying concentrations, measuring their rate of transport on the ciliated epithelium of a frog palate (Majima *et al.*, 1985).

1.1.3.7 Cleft palate

Otitis media with effusion affects 92-97% of children with cleft palate in their first year and persists in 70% at age 4 (Dhillon, 1988; Robinson *et al.*, 1992). The high incidence is caused by impaired muscular compliance of the ET resulting in ineffective opening of the tube (Huang *et al.*, 1997). There is evidence that bacterial infection may play a significant role in the development of OME in children with cleft palate due to 55% of effusions being culture positive (Jousimies-Somer *et al.*, 1986). This is possibly due to the compliance of the ET allowing bacteria to reflux into the middle ear and that due to their inability to suckle, the majority of cleft palate infants are fed on formula milk which does not contain maternal antibodies against middle ear pathogens (Owen *et al.*, 1993; Duffy *et al.*, 1997).

1.1.4 Diagnosis and treatment

The diagnosis of OME is difficult because the disease process is often asymptomatic, there are no signs of acute infection as in AOM such as fever, pain or discharge. It may go undetected by the parent, teacher and even the child. Diagnosis is usually made by physical examination of the ear. Otoscopic examination of the ear of a child with OME reveals the tympanic membrane to be retracted, dull and opaque. The colour of the tympanic membrane can range from light pink to amber and even dark blue (if there has been haemorrhaging). The presence of air bubbles or air-fluid levels makes the diagnosis more evident. Tympanometry is a very useful test for the diagnosis and effective screening of OME. It measures changes in acoustic impedance of the tympanic membrane-middle ear system with changes in air pressure in the external auditory canal.

Various medical and surgical measures have been applied to OME (Fitzgerald *et al.*, 1988; Cantekin and McGuire, 1998; Gilbert, 1999), but no overall single measure is considered to be completely effective (Fraser *et al.*, 1977; van Buchem *et al.*, 1981). This is possibly because OME is prone to both seasonal variation and remission and that many cases of OME resolve spontaneously, therefore it is difficult to assess the cure rate.

Surgical intervention generally involves removal of the effusion by myringotomy and insertion of a grommet into the tympanic membrane. The grommet restores middle ear ventilation, enables aeration of the middle ear and alleviates hearing loss. Grommet insertion is by far the most common short-term measure for treating the symptoms of OME and the most common operation performed on children in the UK (Maw and Bawden, 1993). However, grommet insertion does not offer a cure and the procedure is not free of complications and long-term sequelae related to the middle ear and tympanic membrane, such as cholesteatoma, tympanosclerosis, presence of granulation tissue or ossicular fixation, especially in children who have required grommet insertion on more than one occasion (Moller, 1984; Chopra, 2000)

1.2 Mucus and the upper respiratory tract

1.2.1 Mucus

Mucus is a unique, complex, viscous biological secretion, a mixture of water (95%), proteins and glycoproteins (3%), lipids (1%) and salts (1%) (Creeth, 1978).

Mucus secretions protect the epithelia of mammalian respiratory, gastrointestinal and reproductive tracts and are secreted over the eye. Mucus provides a selective barrier adapted to the specific physiology of each mucosa and its primary function is to protect the underlying mucosal epithelium from damaging agents and shear forces.

In the respiratory tract, including the modified respiratory epithelium of the middle ear, mucus provides a protective barrier against dehydration, invading microorganisms and airborne particles. It is an integral part of the mucociliary escalator and particles trapped in the luminal mucus are constantly removed from the airway and towards the pharynx by ciliary beating. Maintenance of normal mucociliary function depends on the viscoelastic property of mucus, which is determined by the quality and quantity of mucins present in it (Kim *et al.*, 1997). If the mucus becomes too viscous the cilia are not able to move it, not producing enough mucus can cause mucociliary transport to cease. Any abnormalities in mucus secretion may result in impaired clearance and the development of pathological airways. Two frequent pathological changes observed in respiratory epithelium are squamous metaplasia of the mucociliary epithelium and hypersecretion of mucus as a result of hyperplasia of the mucus secreting cells. The latter is a common complication frequently observed in many diseases of the respiratory tract e.g., otitis media, sinusitis and rhinitis. Traditional strategies for controlling mucus

hypersecretion include enhancing the capability of the epithelium to transport mucus using mucoactive drugs such as corticosterone (Miyata *et al.*, 1998) and changing the rheological properties of the mucus using mucolytic agents (Pearson *et al.*, 1997) to reduce mucus viscosity and allow faster removal of the mucus secretion.

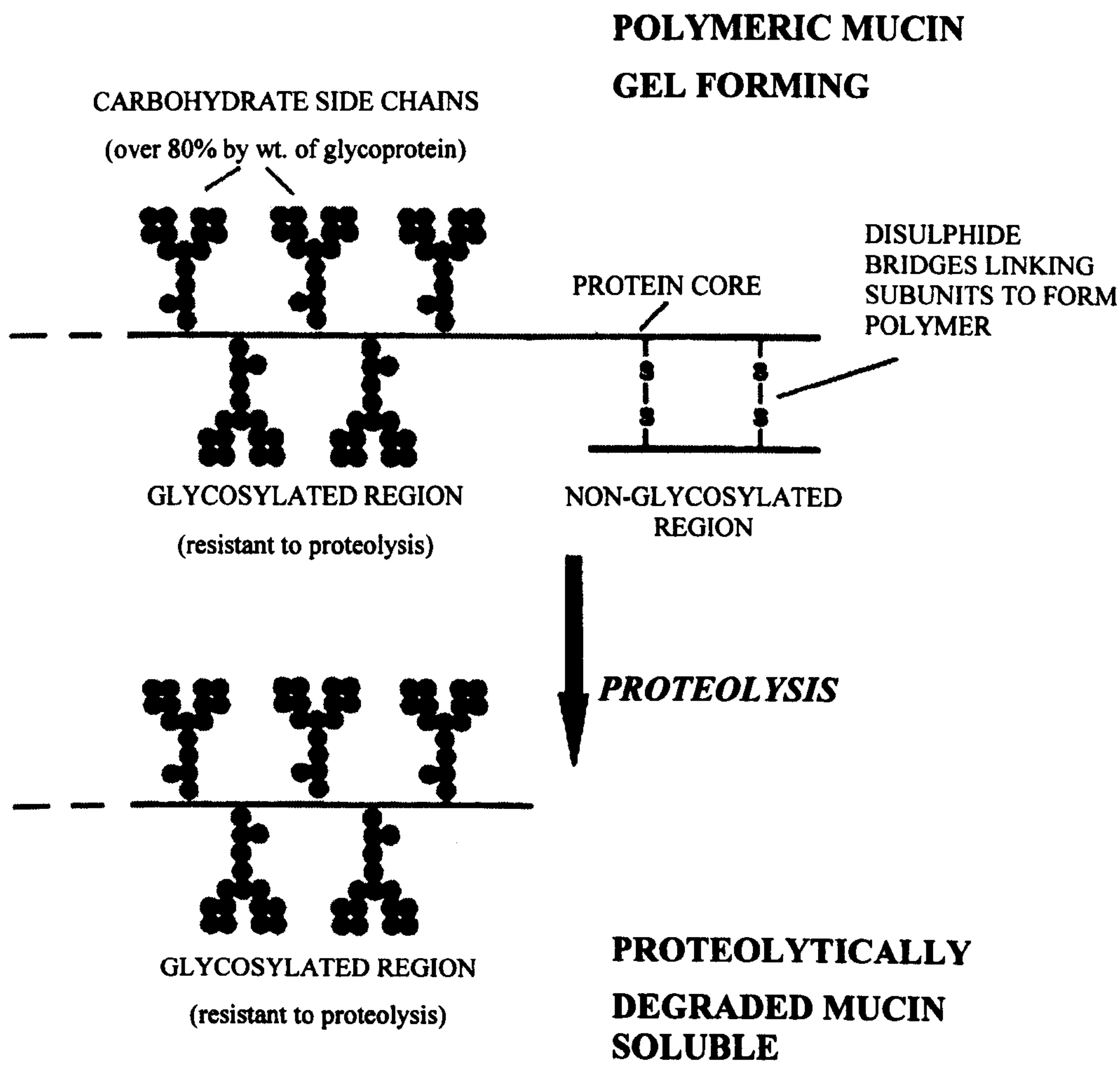
1.2.2 Mucins

Mucus glycoproteins (mucins) are a heterogeneous group of complex, richly glycosylated high molecular weight (approximate $M_r=10^6$ to 50×10^6) (Harding, 1989) molecules, produced by goblet cells of the surface epithelium, submucosal gland and surface mucosal cells.

Mucin subunits are made up of a central protein backbone, 1500 to 4500 amino acids in length, with hundreds of carbohydrate side chains attached (Larivee *et al.*, 1994). There are areas of dense glycosylation (resistant to proteolysis) and areas of little or no glycosylation (susceptible to proteolysis) (Allen and Pearson, 1993) giving a “bottle-brush” structure (Allen *et al.*, 1998) (Figure 1.4). Mucins are difficult to sequence by conventional techniques using enzymes as much of the structure of the protein core is concealed by complex glycosylation, making access difficult.

They form large expanded molecules in solution and interdigitation of the side chains from neighbouring molecules produces the viscous and characteristic gel-like nature of mucus secretions (Sellers *et al.*, 1988a). Pig submaxillary mucin or human tracheobronchial mucin can form a gel at concentrations of $5\text{-}15\text{mgml}^{-1}$ (Sellers *et al.*, 1988b; McCullagh *et al.*, 1995) whereas gastrointestinal mucins form gels at $30\text{-}50\text{mgml}^{-1}$ (Sellers *et al.*, 1988a).

Figure 1.4 The subunit structure of mucus glycoprotein showing glycosylated “bottle brush” and nonglycosylated regions. Subunits are joined by disulphide bridges to form overall linear polymeric mucins. From (Allen, 1989).



1.2.2.1 Protein core structure

The basic unit of a mucin is a single polypeptide chain that forms 10-30% of the weight of the molecule (Fitzgerald *et al.*, 1987; Allen, 1989). The protein core of the mucin subunit can be divided into two regions (Scawen and Allen, 1977):

1. Central protease resistant domains

These domains are heavily glycosylated. They are rich in serine and/or threonine and proline (Allen, 1989) and these amino acids constitute 30-50% of the protein core (Pearson *et al.*, 1980; Carlstedt *et al.*, 1983; Forstner and Forstner, 1994). This part of the mucin contains repetitive regions (Allen *et al.*, 1998) including tandem repeat domains varying in length and sequence from 8 to 169 residues (Toribara *et al.*, 1993; Guyonnet-Duperat *et al.*, 1995) and is also known as the variable number tandem repeat region (VNTR).

2. Terminal protease sensitive domains

These domains are virtually devoid of carbohydrate. They are rich in cysteine and acidic groups. Cysteine is involved in the formation of disulphide bridges between the subunits of the polymer and is therefore important in determining the viscous properties of the mucin (Allen and Pearson, 1993).

1.2.2.2 Carbohydrate structures

Carbohydrate forms up to 85% of the mucin molecule, the main sugars being fucose, galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine and sialic acid (Pigman, 1977; Creeth, 1978; Allen, 1983). The absence of uronic acid and only trace amounts of mannose (<1%) distinguishes mucin glycoproteins from the proteoglycans of connective tissue and serum glycoproteins respectively.

The carbohydrate side chains vary in composition, structure and size between mucin species from different secretions. They form branching chains of between 2 and 20 sugars (Forstner and Forstner, 1994). In the central VNTR region of the mucin subunit they are attached covalently to the serine and threonine by alkali labile *O*-glycosidic linkages from *N*-acetylgalactosamine at their reducing ends (Allen and Pearson, 1993), protecting the protein from proteolytic attack. *N*-acetylgalactosamine always occurs as the linkage sugar to the peptide core (Carlson, 1977).

The backbone region of the oligosaccharide chain is composed of alternating residues of β -linked galactose or *N*-acetylgalactosamine. Sialic acid and fucose, and sometimes *N*-acetylgalactosamine, are found at the non-reducing ends of the main or branched chains. The terminal sugars confer ABO blood group activity on the mucins due to the structure of the non-reducing ends of their oligosaccharide chains (Allen, 1989).

The cysteine-rich regions of the mucins at the N- and C-terminals contain *N*-glycosylation sites. *N*-glycosylation is thought to be a key signal for subcellular targeting of nascent mucin glycoprotein since inhibition of this process arrests biosynthesis (McCool *et al.*, 1994).

Throughout their length, the carbohydrate chains have sulphate groups attached via ester linkages to galactose and *N*-acetylgalactosamine residues. These and sialic acid residues impart a negative charge to the molecule (Forstner and Forstner, 1994) leading to acidic mucins rather than fucose-containing neutral mucins (Allen and Leonard, 1985). Histological staining such as Periodic acid Schiffs (PAS)-Alcian blue and high iron diamine can differentiate between acidic and neutral mucins (Filipe, 1979).

1.2.3 Mucin genes

To date, 18 human mucin genes coding for mucin protein cores have been identified and assigned to the *MUC* gene family as approved by the Human Genome Organization Gene Nomenclature Committee. They are divided into two types, secretory and membrane associated (Gendler and Spicer, 1995).

Secretory mucins contribute to the viscous mucus of the respiratory, gastrointestinal and reproductive tracts. They typically form very large oligomers through the linkage of glycoprotein monomers. The glycoproteins are secreted from the cell and remain at the apical surface of epithelial cells in the form of a mucus gel.

Membrane associated mucins have a hydrophobic, membrane-spanning domain and have not been observed to form oligomeric complexes (Figure 1.5). They form part of the cell-surface glycocalyx and can protect epithelial cells against pathogens or from being injured by inflammatory cells through preventing them from binding to the cell surface.

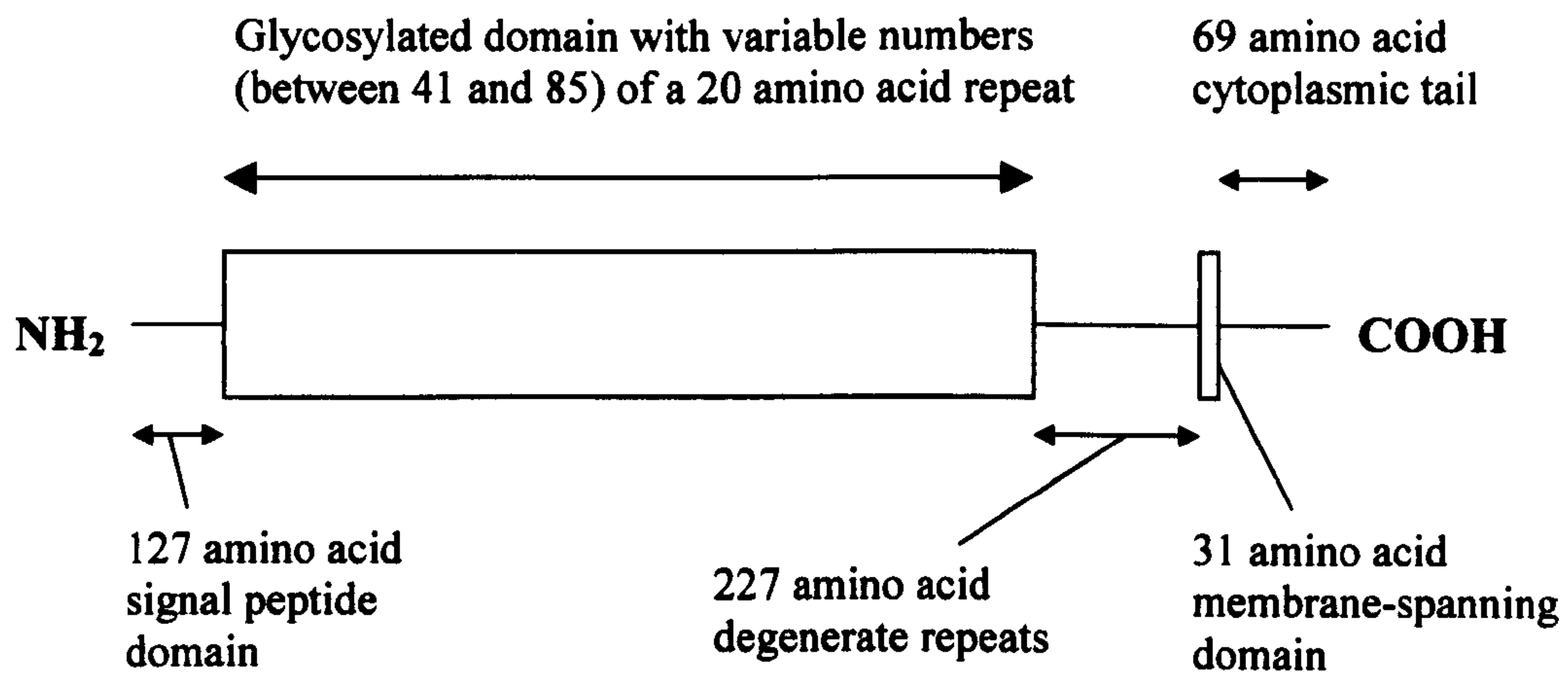
Of the secreted mucins four, MUC2, MUC5AC, MUC5B and MUC6 are known to be gel forming (Desseyn, 2000) and their four genes are contained within a single 400kb genomic DNA fragment on chromosome 11 band p15.5 (Pigny *et al.*, 1996). Each has a large central tandem repeat region and the amino and carboxyl terminals of the protein cores of these mucins are structurally related to each other (Figure 1.6). The cDNA sequences flanking the central VNTR region for MUC2, MUC5AC and MUC5B code for cysteine-rich domains designated B, C, CK (cysteine knot) and D (D1, D2, D', D3 and D4) domains, which are similar to the cysteine-rich A-domain flanking regions in the serum glycoprotein prepro-von Willebrand factor (vWF), a blood-clotting factor

(Gum Jr *et al.*, 1992; Klomp *et al.*, 1995; Lesuffleur *et al.*, 1995; Buisine *et al.*, 1998; Desseyn, 2000) (Figure 1.7, 1.8). MUC6 is structurally remote as a large portion of the region downstream of the VNTR has been lost during evolution (Toribara *et al.*, 1997). The D domains present in all these secreted mucins are proposed to be involved in the dimerisation of mucin molecules (Gum Jr, 1995; Gipson *et al.*, 2001).

Several membrane-bound mucins are also related to each other, especially those localized to chromosomal locus 7q22, MUC3A, MUC3B, MUC11, MUC12 and MUC17 (Williams *et al.*, 1999; Pratt *et al.*, 2000; Gum Jr *et al.*, 2002).

The predominant feature of all the mucin genes is the presence of tandem repeats of defined sequences of nucleotides (this has not been confirmed as yet for MUC18). There is no homology between the tandem repeat sequences of the mucin genes. Each mucin also has distinct tissue expression that can be determined by detection of mRNA and also the protein product (Audie *et al.*, 1993; Gendler and Spicer, 1995; Ho *et al.*, 1995; Van Klinken *et al.*, 1995). The tissues in which each mucin is expressed and the chromosomal location of the genes are shown in Table 1.1.

Figure 1.5 Diagrammatic representation of the structure of a membrane-bound mucin, MUC1 (adapted from (Gendler and Spicer, 1995; Gum Jr, 1995))



MUC1 is over 1300 amino acid residues in length and consists of three distinct segments. The amino terminal signal peptide domain consists of a signal peptide and degenerate repeats. There is a large glycosylated domain made up of variable numbers of a 20 amino acid repeat which is less glycosylated than MUC2 (Fig. 1.8) due to a lower density of potential *O*-glycosylation sites. The carboxyl terminal consists of degenerate repeats containing potential *N*-glycosylation sites, a hydrophobic membrane-spanning domain of 31 amino acids, which serves to anchor the mucin to the membrane and a cytoplasmic domain of 69 amino acids.

Figure 1.6 The hypothetical evolution of the four human mucin genes clustered on the chromosome 11p15 from a common ancestor of the human *vWF* gene (from (Desseyn, 2000)).

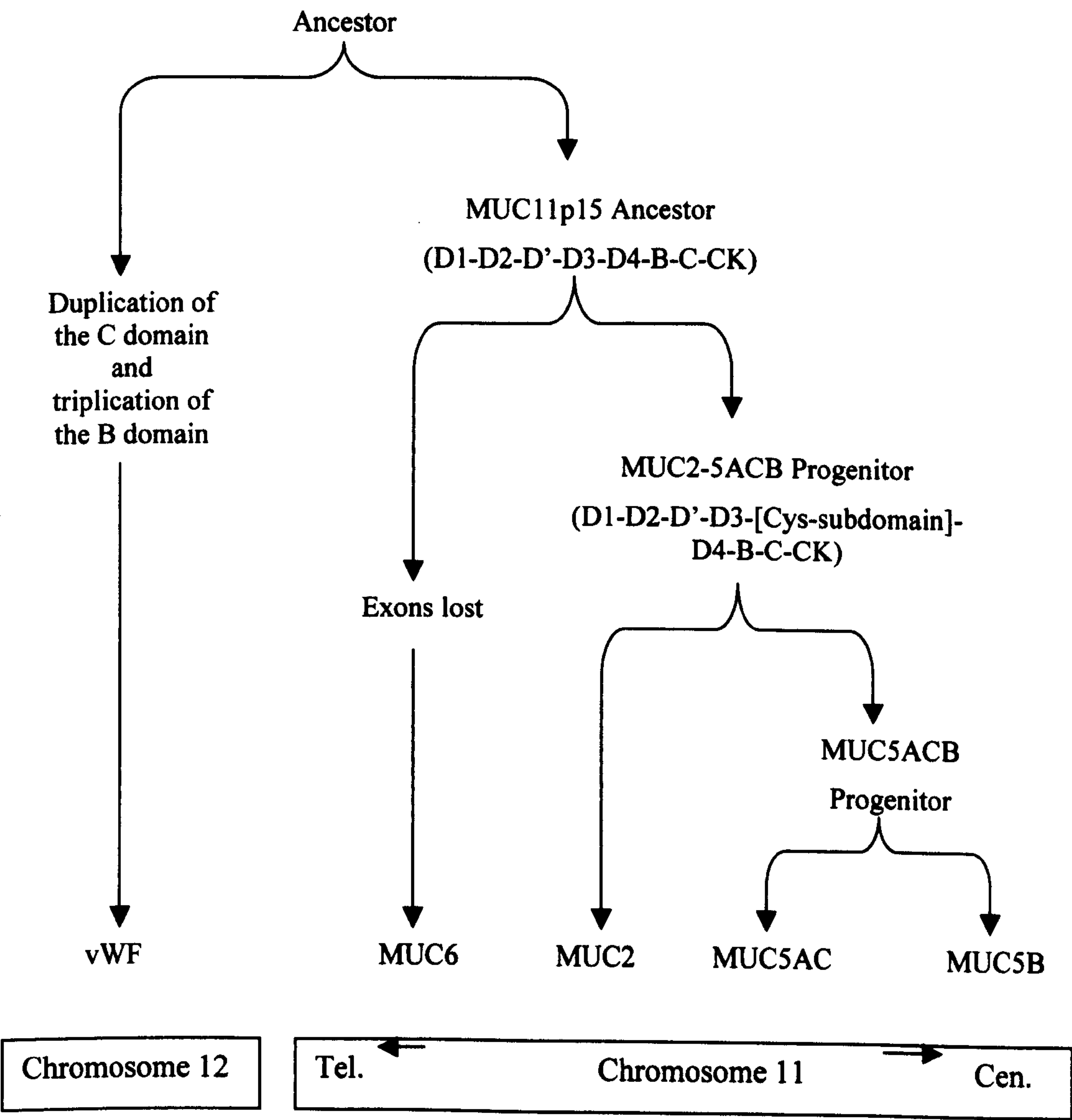
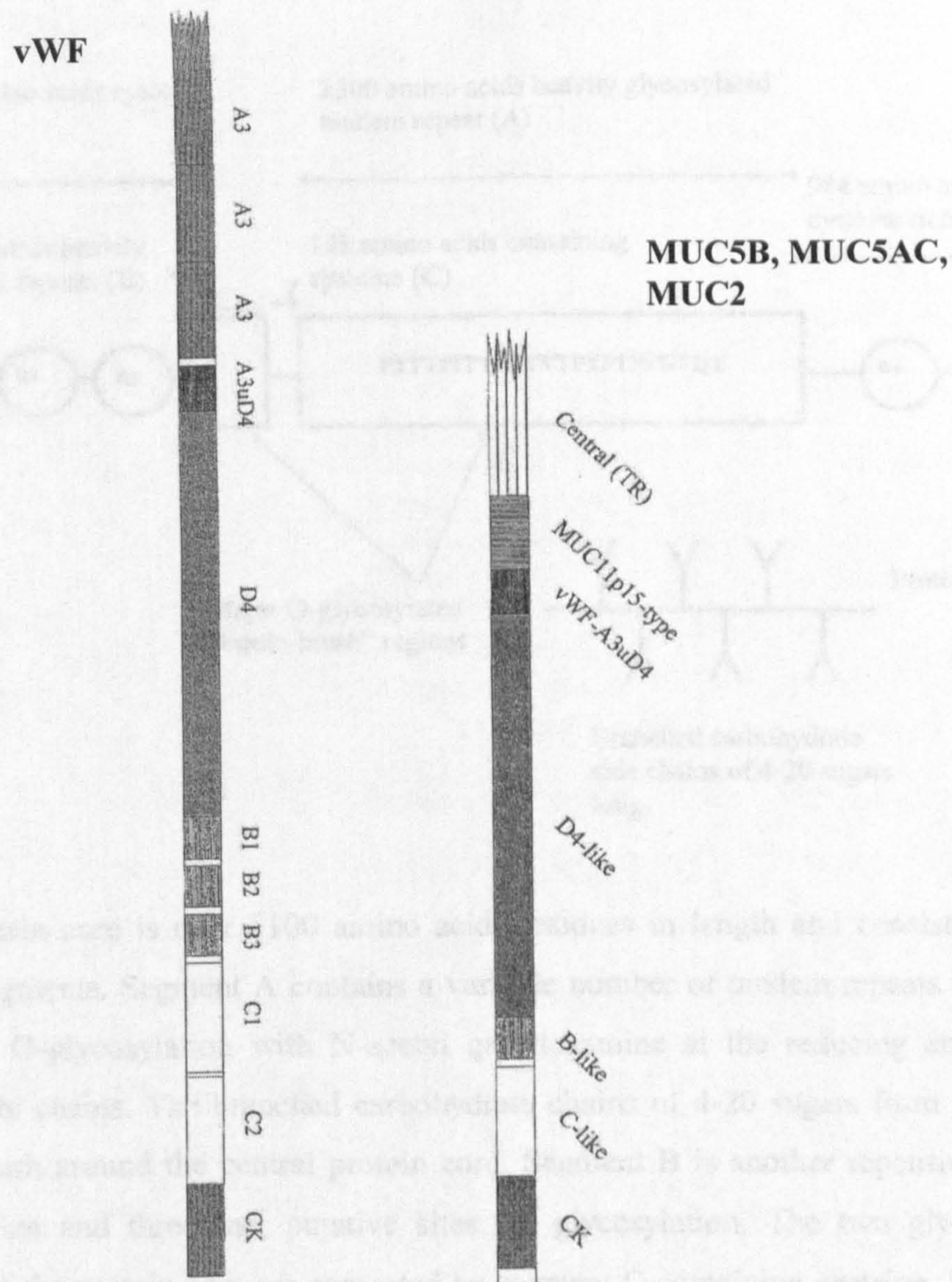
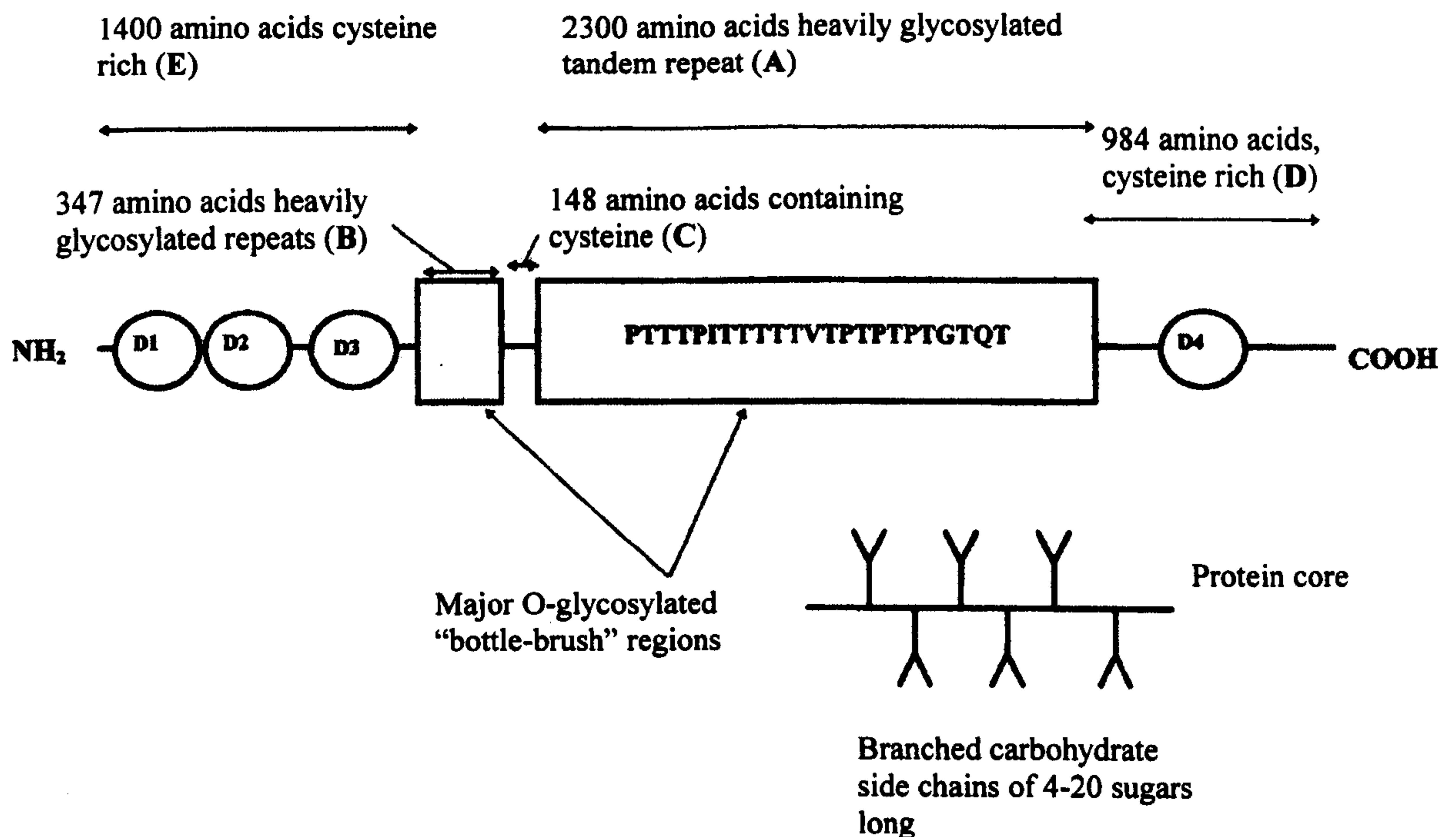


Figure 1.7 A schematic diagram of the carboxyl terminal MUC5B core protein (and similar proteins) and von Willebrand factor (Desseyn *et al.*, 1997a)



The conservation of nearly all the cysteine residues and of several other amino acids of vWF and of the three 11p15.5 human mucins MUC2, MUC5AC and MUC5B is readily apparent, suggesting a very similar tertiary structure that can be dissected into six domains. The MUC11p15-type domain follows the central exon, the A3uD4 domain has similarities to the domain located between A3 and D4 in vWF, and there is one D4-like domain, one B-like domain, one C-like domain and one CK domain.

Figure 1.8 Diagrammatic representation of the structure of a secreted mucin, MUC2 (Allen *et al.*, 1998)



MUC2 protein core is over 5100 amino acids residues in length and consists of five different segments. Segment A contains a variable number of tandem repeats (VNTR), the site of O-glycosylation with N-acetyl galactosamine at the reducing end of the carbohydrate chains. The branched carbohydrate chains of 4-20 sugars form a closely packed sheath around the central protein core. Segment B is another repetitive region rich in serine and threonine, putative sites for glycosylation. The two glycosylated segments of the protein core are separated by segment C containing cysteine. Segments D and E at the C and N terminal ends, respectively, are extensive peptide chains, rich in cysteine and containing D domains which have sequence homology to the prepro vWF. Segments C, D and E contain 31 potential N-glycosylation sites and segment D contains a cysteine 'knot' of eleven conserved cysteine residues found in the other known secretory mucin gene products (MUC5AC, MUC5B and MUC6).

Table 1.1 Tissue expression and chromosomal location of the 18 mucin genes.
S = secretory mucin, M = membrane bound.

Mucin gene	Chromosomal location		Tissue expression	References
MUC1	1q21-23	M	All epithelia, breast, pancreas, small intestine, urinary bladder	(Russell <i>et al.</i> , 1998)
MUC2	11p15.5	S	Airways, tear ducts, small intestine, colon	(Griffiths <i>et al.</i> , 1990; Gum Jr <i>et al.</i> , 1992)
MUC3A	7q22	M	Gall bladder, small intestine, colon	(Pratt <i>et al.</i> , 2000)
MUC3B	7q22	M	As 3A plus heart, liver, thymus, pancreas	(Pratt <i>et al.</i> , 2000)
MUC4	3q29	M	Trachea, lung, stomach, colon, uterus, prostate, small intestine	(Moniaux <i>et al.</i> , 1999)
MUC5AC	11p15.5	S	Middle ear, airways, stomach, cervix	(Hovenberg <i>et al.</i> , 1996; Labat <i>et al.</i> , 1999)
MUC5B	11p15.5	S	Middle ear, airways, submaxillary gland, endocervix, gall bladder, pancreas	(Desseyn <i>et al.</i> , 1997b; Kawano <i>et al.</i> , 2000)
MUC6	11p15.5	S	Stomach, gall bladder, cervix	(Toribara <i>et al.</i> , 1993)
MUC7	4q13-21	S	Salivary glands, airways	(Bobek <i>et al.</i> , 1993)
MUC8	12q24.3	M	Airways	(Shankar <i>et al.</i> , 1997)
MUC9	1q13	M	Oviducts	(Lapensee <i>et al.</i> , 1997)
MUC11	7q22	M	Colon	(Williams <i>et al.</i> , 1999; Moniaux <i>et al.</i> , 2001)
MUC12	7q22	M	Colon	(Williams <i>et al.</i> , 1999; Moniaux <i>et al.</i> , 2001)
MUC13	3q13	M	Airway columnar epithelium and goblet cells, colon	(Williams <i>et al.</i> , 2001)
MUC15	11p14	M	Tonsil, breast, thymus, adult spleen, prostate, ovary, small intestine, colon	(Pallesen <i>et al.</i> , 2002)
MUC16	19p13.3	M	Ovarian epithelia	(Yin and Lloyd, 2001)
MUC17	7q22	M	Trachea, lung, pancreas, colon, small intestine	(Gum Jr <i>et al.</i> , 2002)
MUC18	11q23	M	Cell adhesion molecule - prostate	(Wu <i>et al.</i> , 2001a; Wu <i>et al.</i> , 2001b)

The following mucin genes have been shown to be expressed by human middle ear mucosa using *in situ* hybridisation, MUC1, MUC2, MUC4, MUC5AC, MUC5B and MUC7 (Hutton *et al.*, 1998b; Severn *et al.*, 1999). The expression of a mucin gene does not necessarily lead to secretion of the product however, as no MUC2 has been reported in middle ear effusions (Hutton *et al.*, 1998a).

1.2.3.1 MUC1

MUC1 is a transmembrane protein present in the apical plasma membrane of epithelial cells (Pemberton *et al.*, 1992). MUC1 was the first mucin core to be cloned and full-length cDNA and genomic clones for human *MUC1* have been published (Gendler *et al.*, 1990). The cDNA sequence of *MUC1* encodes a protein of over 1300 amino acids (Figure 1.5) and is contained in seven exons spanning between four and seven kb pairs depending on the number of repeat units present (Lancaster *et al.*, 1990). MUC1 exhibits polymorphism and the core protein can be glycosylated in different ways therefore the mature glycosylated forms appear distinct and the mucin is not of a specific molecular mass and structure. MUC1 from the mammary gland has a M_r of 250 to 500kDa and is 50% carbohydrate (Shimizu and Yamauchi, 1982) and MUC1 from the pancreas has a molecular weight over 1000kDa and is 80% carbohydrate (Lan *et al.*, 1987).

1.2.3.2 MUC2

MUC2 was the first human secretory mucin to be cloned and completely sequenced (Gum Jr *et al.*, 1992; Gum Jr *et al.*, 1994; Gendler and Spicer, 1995). The sequence is contained in 15.6kb pairs and codes for a large secreted protein over 5100 amino acids in length (Gum Jr *et al.*, 1994) (Figure 1.8). The *MUC2* gene is highly repetitive,

comprising of two repeat domains, one a tandem repeat region with approximately 100, fairly precise, 23 amino acid repeats and the second with a 347 amino acid irregular repeat (Toribara *et al.*, 1991). Cysteine-rich subdomains are located upstream and downstream of the central repeat region and have sequence similarity with vWF, suggesting that these domains are important in the end to end polymerisation of subunits via disulphide bridges resulting in polymeric mucin molecules (Gum Jr *et al.*, 1992).

1.2.3.3 *MUC4*

The genomic organisation of *MUC4* has been determined. The 5' region and the central part of the *MUC4* gene were characterised first (Nollet *et al.*, 1998). The central, tandem repeat domain varies from 2334–6334 amino acids due to VNTR polymorphism with repeats of 16 amino acid residues. The C-terminal region contains a hydrophobic transmembrane domain and a short cytoplasmic tail. The 3' structure has more recently been determined (Escande *et al.*, 2002) and is shown to have an extended structure that would suggest that the mucin is present above the cell membrane far higher than the other membrane-associated proteins. It is possible that a *MUC4* complex could be involved in a signalling pathway that is required for proliferation and differentiation of epithelial cells (Moniaux *et al.*, 1999).

1.2.3.4 *MUC5B*

The *MUC5B* gene is 40.1kb in length with 49 exons encoding a 5701 amino acid peptide with a M_r of approximately 627,000. The structural organisation of the peptide deduced from the nucleotide sequence of the central region of *MUC5B* has been determined (Desseyn *et al.*, 1997b). It is a single large exon of 10713 base pairs containing the entire tandem repeat domain and is the biggest described for a vertebrate

gene. It codes for a 3570 amino acid peptide and 19 subdomains have been identified. Both the 3' (Desseyn *et al.*, 1997a) and 5' (Van Seuningen *et al.*, 2000) flanking regions of MUC5B have been characterised and have cysteine-rich domains similar to the other genes present on chromosome 11p15.5 (MUC2, MUC5AC and MUC6) and with vWF.

1.2.3.5 MUC5AC

MUC5AC has been partially characterised. The organisation and complete nucleotide sequence of the 3' end of the *MUC5AC* gene is known (Meerzaman *et al.*, 1994; Lesuffleur *et al.*, 1995; Buisine *et al.*, 1998). The tandem repeat domain is 8 amino acid residues in length, this is much shorter than that of MUC5B (Guyonnet-Duperat *et al.*, 1995). More recently, the amino terminal and 5' flanking region of the MUC5AC mucin gene has been isolated (Li *et al.*, 1998) and the full length of MUC5AC mRNA is approximately 12-14kb. The comparative molecular analysis of *MUC5AC* and the other genes of the 11p15.5 gene family reveal common cysteine-rich domains supporting the idea that they have evolved from a common ancestral gene (Buisine *et al.*, 1998).

1.2.3.6 MUC7

MUC7 has been fully characterised (Bobek *et al.*, 1993) and encodes for a protein of 357 amino acid residues with a M_r of 37kDa. The MUC7 peptide does not polymerise, an exception among the secreted mucins. The *MUC7* gene encodes for the protein core of low molecular weight mucin (MG2) in saliva. The VNTR region is composed of six tandem repeats of 23 amino acid residues (Bobek *et al.*, 1993) and is heavily glycosylated (Gururaja *et al.*, 1998). There is a minimal degree of VNTR polymorphism (Bobek *et al.*, 1996).

In summary, MUC1 and MUC4 are membrane bound mucins and possess no structural domains. They are involved in mucosal protection. MUC2, MUC5AC and MUC5B are polymeric secreted mucins with cysteine-rich regions homologous to each other and to von Willebrand Factor. MUC7 is a monomeric secreted mucin with no structural homology to any of the other secreted mucins.

1.2.4 Control regions in mucin genes for upregulation of expression

Increased mucin gene expression has been shown to occur via two mechanisms; by upregulation of *MUC* transcription (Basbaum *et al.*, 1999) and/or by mRNA stability (Rose *et al.*, 2000).

Most *in vitro* studies have focused on the regulation of *MUC2* and *MUC5AC* genes by various mediators. The regulation of *MUC5B* has not been as well investigated, but it has been demonstrated that several mediators that increase *MUC5AC* expression (such as acrolein, an aldehyde present in tobacco smoke) have no effect on *MUC5B* (Borchers *et al.*, 1999). It has been demonstrated *in vitro* that different cytokines have different effects on the stimulation of mucin secretion (Smirnova *et al.*, 2001; Smirnova *et al.*, 2002), which is possibly due to each cytokine having a particular signal transduction pathway (Taga *et al.*, 1989; Wiegmann *et al.*, 1992) and that induction of secretion of *MUC5AC* appears to be easier than *MUC5B*. The amount of protein generated from each mucin transcript can be varied by message stabilisation. Mucin mRNA stabilisation has been documented following the treatment of cells with cytokines (Borchers *et al.*, 1999; Rose *et al.*, 2000) and this could lead to more efficient translation of the message, increasing gene expression.

Recent studies of the 5' flanking regions of MUC5AC and MUC5B genes have identified nuclear factor κ B (NF- κ B) binding sites within the functional promoter regions for both genes (Li *et al.*, 1998; Van Seuningen *et al.*, 2000). NF- κ B is a transcription factor present in virtually all cells. It regulates the transcription of an exceptionally large number of genes, especially those involved in the immune and inflammatory responses. TNF- α operates through this mechanism and so MUC5AC and MUC5B should both be upregulated in the presence of this cytokine. *Cis*-elements have been found in the 5' flanking region of *MUC5B* (Chen *et al.*, 2001), which are potentially involved in the regulation of MUC5B gene expression by steroids such as retinoic acid (De Bolos *et al.*, 1998) and regulate the growth and differentiation of tracheobronchial epithelial cells including mucin gene expression (Gray *et al.*, 2001).

The *MUC5AC* and *MUC5B* genes are present in the same cluster at chromosomal location 11p15.5 (Pigny *et al.*, 1996) and have common sites for transcription factors within functional promoter regions (Li *et al.*, 1998; Van Seuningen *et al.*, 2000) yet due to differences observed in the induction of the two mucin genes by cytokines, they are unlikely to be controlled in the same manner, which may lead to their involvement in different stages of the inflammatory process. The mechanisms of signalling systems that lead to increased transcription of mucin genes are still to be elucidated.

1.2.5 Mucin gene expression in the middle ear

The middle ear epithelium is modified respiratory in type (Lin *et al.*, 2001), however very little is known about mucin gene expression in the normal middle ear due to difficulties in obtaining normal human tissues.

In situ hybridisation studies on middle ear biopsies taken from patients undergoing middle ear surgery showed MUC1, MUC2, MUC4, MUC5AC, MUC5B and MUC7 to be expressed by human middle ear mucosa (Severn *et al.*, 1999). MUC1, MUC2, MUC4 and MUC5AC appeared to be expressed by surface epithelial cells whereas MUC5B and MUC7 expression was in glandular epithelial cells. The tissue expression of the mucin genes in middle ear mucosa is consistent with mucin gene expression patterns in other tissues using Northern blot analysis (Severn *et al.*, 1997), *in situ* hybridisation and reverse transcriptase PCR (Takeuchi *et al.*, 1995; Aust *et al.*, 1997). MUC3 and MUC6 were not found in the middle ear mucosa. This is not surprising for MUC6 expression, which has never been identified in respiratory epithelia (Biesbrock *et al.*, 1995).

Middle ear effusions contain at least two different populations of mucin molecule, coded for by *MUC5B* and *MUC5AC* (Hutton *et al.*, 1998a; Kawano *et al.*, 2000; Lin *et al.*, 2001). This is supported by evidence of MUC5AC protein in middle ear goblet cells and MUC5B protein in submucosal mucus glands using relevant specific antisera by immunohistochemistry (Hutton *et al.*, 1998b; Chung *et al.*, 2002).

In one study by Hutton (Hutton *et al.*, 1998a), mucins from human middle ear effusions were isolated and characterised in order to determine the mucin gene products secreted by the middle ear mucosa. Up to 15% of thick (and 5% of thin) middle ear effusions

contained MUC5AC and epitopes present on MUC5B were also present in mucins in the effusions suggesting that MUC5B may also be present.

Kawano (Kawano *et al.*, 2000) studied middle ear mucosa specimens from patients undergoing surgical procedures for chronic otitis media (present for three months or more). Using Alcian blue–PAS staining of mucosal sections it was observed that the epithelium was highly secretory and that the apical layer and gland-like structures were abundant in glycoconjugates and the submucosal layer was rich in inflammatory cells. *In situ* hybridisation and immunohistochemistry studies demonstrated that gland-like structures of the middle ear submucosa in chronic otitis media expressed MUC5B mucin mRNA and their product, MUC5B mucin. It was clear in this study that expression of the MUC5B mucin gene was related to infiltration of inflammatory cells in the submucosa and it was therefore suggested that inflammatory cell products (such as cytokines) are involved in the production of MUC5B by upregulating expression of the MUC5B mucin gene.

In a recent study by Lin (Lin *et al.*, 2001), mucin gene expression in the middle ear of patients with chronic otitis media was compared to the normal middle ear and a pool of middle ear effusions was also analysed. *In situ* and Northern blotting studies demonstrated expression of MUC5B in both the ‘normal’ and inflamed ear however the gene expression was 4.2 fold higher in the inflamed ear. MUC4 gene expression was detected only in the inflamed ear and at lower levels than MUC5B. MUC5AC was not detected at all. The upregulation of mucin gene expression was accompanied by an increase of MUC5B- and MUC4-producing cells in the middle ear mucosa and the presence of MUC5B and MUC4 mucin in middle ear effusions. These results were indicative of a transition from a normal to a hypersecretory epithelium. The mechanism

of upregulation of mucin genes is not currently understood but an insight into the mechanisms could lead to the development of intervention strategies against mucus hyperproduction in chronic otitis media.

Various *in situ* hybridisation studies have shown more than one mucin gene to be expressed by the epithelial cells in the same tissue making it likely that mucus secretions are a carefully controlled balance of mucin gene products. If this balance is disturbed in disease, the result could be secretions that are more or less viscous than normal. In OME, the viscosity of a secretion can radically affect the role it plays, with a protective role in the normal situation replaced by a pathological role when mucin secretion is up regulated in the diseased state. Changes in mucin gene expression have been demonstrated in metaplastic epithelia (Kim *et al.*, 1996) and mucus cell metaplasia in middle ear epithelium is the key event leading to mucus hyperproduction (Kawano *et al.*, 2000).

It is important to identify the factors or mediators involved in the change from a normal middle ear epithelium into a highly secretory epithelium and in the regulation of mucin gene expression.

1.3 Gastro-oesophageal reflux in children

1.3.1 Gastro-oesophageal reflux

Gastro-oesophageal reflux (GOR) is the retrograde movement of gastric contents through the lower oesophageal sphincter (LOS) into the oesophagus. It is caused by transient and intermittent LOS relaxation to a zero gradient between the gastric and oesophageal lumens (Hart, 1996; Zalzal and Tran, 2000) unrelated to swallowing. GOR is generally classified into four types (Zalzal and Tran, 2000).

1. Physiologic

Episodic reflux resulting in infrequent vomiting is observed with no abnormalities on any diagnostic study. It is common in neonates and infants and decreases during the first year of life (Vandenplas *et al.*, 1991). This type of reflux is believed to occur in approximately 67% of children at four months of age with resolution in most by ten to twelve months (Nelson *et al.*, 1997)

2. Functional

Silent or symptomatic reflux, observed in children who exhibit daily vomiting where there is no morbid condition. This can be confirmed by intra-oesophageal pH monitoring.

3. Pathologic

A more severe form of functional reflux, which can interfere with the normal growth process or cause gastrointestinal disorders and respiratory alterations. Symptoms include epigastric discomfort, regurgitation, heartburn and vomiting. If symptoms

increase in severity this can lead to iron-deficient anaemia, failure to thrive and oesophagitis (Herbst, 1981; Contencin and Narcy, 1991; Zalzal and Tran, 2000).

4. Secondary GOR

This is related to other deficits such as neurological anomalies (including incoordination of swallowing and pooling of secretion within the hypopharynx), hiatal hernia (associated with LOS incompetence) or Barrett's oesophagus (gastric metaplasia of the oesophagus due to acid exposure) (Orenstein *et al.*, 1999) and may require surgery to prevent morbidity or mortality.

Gastric contents may contain (as well as gastric acid and pepsin) bile salts and pancreatic enzymes (Hart, 1996), which have the ability to irritate or injure tissues not adapted to the presence of these potentially noxious materials. There are defence mechanisms present in the lumen and body of the oesophagus, such as secondary peristalsis, salivary and oesophageal bicarbonate and mucosal tight junctions that help to prevent significant damage.

Damage to tissues resulting from GOR is termed gastro-oesophageal reflux disease (GORD). GORD is a common disease of infancy and is the most frequently referred condition to a paediatric gastroenterologist (Orenstein *et al.*, 1999; Carroll *et al.*, 2002). GORD has a wide spectrum of expression ranging from symptomatic reflux (where symptoms may be marked despite virtually normal amounts of acid reflux, this can depend on the individuals sensitivity to the reflux contents (Koufman, 1991)) to severe tissue damage.

1.3.2 Diagnosis

The preferred gold standard method of diagnosing reflux is by prolonged ambulatory dual probe pH monitoring (Koufman, 1991; Smit *et al.*, 2000). The goal of this method is the continuous measurement of pH while in a normal-life resembling situation. A catheter with two (or more) electrodes is guided through the nose into the oesophagus, connected to a recorder. For distal pH monitoring, an electrode is placed 5cm above the manometrically determined LOS. Every 4-8 seconds, pH measurements are taken and stored. For more proximal pH monitoring, a second electrode is placed in the hypopharynx, 2cm above the upper oesophageal sphincter (UOS) (Figure 1.9).

Many parameters can be measured using ambulatory pH monitoring:

- Number of reflux episodes ($\text{pH} < 4$)

- Percentage of time that pH is < 4

- Number of reflux episodes of at least 5 minutes duration

- Average oesophageal clearance time per reflux episode

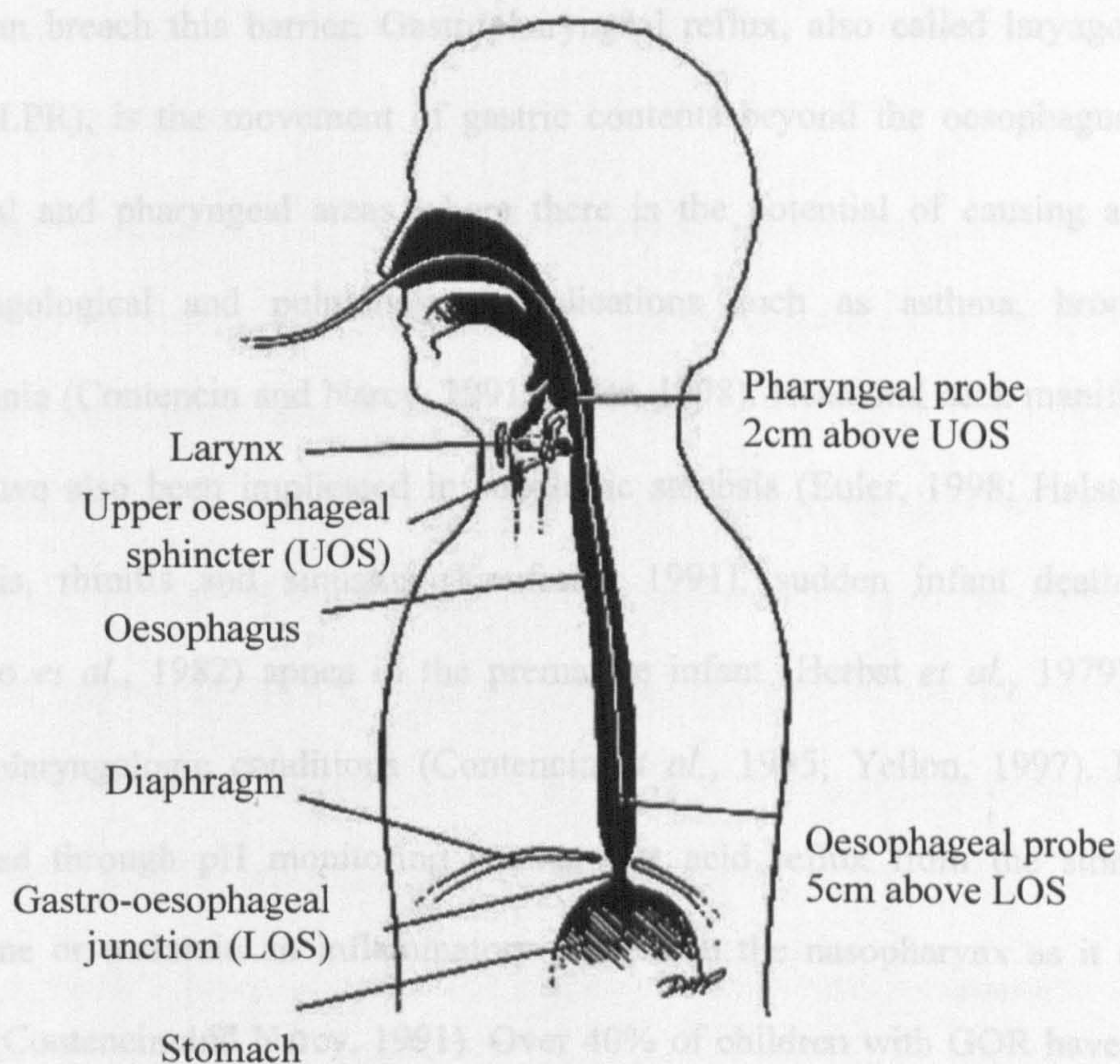
- Longest reflux episode

- Total time of recorded $\text{pH} < 4$

GOR is diagnosed if the oesophageal pH is lower than pH4 for more than 4% of the recording time. A drop below pH4 at the pharyngeal probe preceded by a precipitous drop at the oesophageal probe is considered evidence of laryngopharyngeal reflux (LPR) (Koufman, 1991).

Ten or fewer episodes of pharyngeal reflux may be physiological in children less than one year of age (Halstead, 1999).

Figure 1.9 Technique of double-probe ambulatory intra-oesophageal and pharyngeal pH monitoring (Koufman, 1991)



1.3.3 Extra-oesophageal manifestations

Symptoms of GOR are usually limited to the oesophagus due to the effectiveness of the UOS in preventing reflux to the oropharynx and beyond but under certain circumstances GOR can breach this barrier. Gastropharyngeal reflux, also called laryngopharyngeal reflux (LPR), is the movement of gastric contents beyond the oesophagus up to the laryngeal and pharyngeal areas where there is the potential of causing a variety of otolaryngological and pulmonary complications such as asthma, bronchitis and pneumonia (Contencin and Narcy, 1991; Euler, 1998). Head and neck manifestations of GOR have also been implicated in subglottic stenosis (Euler, 1998; Halstead, 1999), laryngitis, rhinitis and sinusitis (Koufman, 1991), sudden infant death syndrome (Ariagno *et al.*, 1982) apnea in the premature infant (Herbst *et al.*, 1979) and other otorhinolaryngologic conditions (Contencin *et al.*, 1995; Yellon, 1997). It has been suggested through pH monitoring studies that acid reflux from the stomach could determine or maintain an inflammatory process in the nasopharynx as it does in the larynx (Contencin and Narcy, 1991). Over 40% of children with GOR have respiratory manifestations (Andze *et al.*, 1991) and not uncommonly, respiratory disorders are the only presenting symptoms in reflux patients (Stiegmann *et al.*, 1987; Bouchard *et al.*, 1999; Zalzal and Tran, 2000).

It has been speculated that GOR is involved in the pathogenesis of OME (Shilkin, 1994; Halstead, 1999; Velepich *et al.*, 2000) and the anatomic and immunologic immaturity of the Eustachian tube is an explanation for this (Bluestone and Klein, 1996).

1.3.4 Laryngopharyngeal reflux and OME

Due to age-related differences in the length and angle of the ET (13–18mm in length and 10° to the horizontal in children compared to 31–38mm and 45° to the horizontal in adults (Proctor, 1967)), it is more likely that secretions can reflux into the middle ear from the nasopharynx in infants.

The tensor veli palatini (TVP) muscle is responsible for the ventilation of the middle ear and is superiorly orientated relative to the ET lumen in adults due to the angle of the ET. This angulation means that the ventilation mechanism of the ET is more efficient in adults than in infants and young children. Obstruction (opening failure) and abnormal patency (closing failure) are two types of ET dysfunction that can predispose to OME. Abnormal patency of the ET, when the tube is open even at rest, can allow unwanted secretions from the nasopharynx, including reflux contents, to gain access to the ET and to the middle ear cleft due to the short length of the tube.

With age, the ET lumen becomes wider and has an increased volume and is capable of ventilating the middle ear and clearing secretions more effectively. These functional changes occur at an age when the prevalence of childhood OME decreases (Sadler-Kimes *et al.*, 1989) leading to the conclusion that the anatomic differences may be a predisposing factor for the development of OME in children.

Reflux of barium into the nasopharynx, ET and middle ear (Pinckney and Currarino, 1980) was observed during upper gastrointestinal examination of four infants, three with GOR and one with incoordination of swallowing. The presence of acid reflux in the nasopharynx has been documented (Contencin and Narcy, 1991) from a 24-hour continuous nasopharyngeal pH monitoring study in infants and children and a possible relationship between OME and GOR was observed by Velepich *et al.* (Velepich *et al.*,

2000) in a continuous oesophageal pH monitoring study of children with either OME lasting four months or more (n=16) or recurrent otitis media with more than five episodes a year (n=14). Pathological GOR was diagnosed in 60% of the patients, nine from each group and there was no statistical significance between the two types of disease. None of these patients had complained of GOR symptoms. These studies demonstrate that a relationship may occur between GOR and OME and it is clear that further studies are necessary.

If gastric contents (including pepsin and acid) are refluxed into the ET and middle ear cleft there are two possible scenarios, one involving a single reflux episode and bacteria and the other multiple reflux episodes:

1. A single reflux episode may cause damage and inflammation, leading to the production of an effusion that may occlude the immature ET lumen due to its narrow tubular configuration (Suzuki *et al.*, 1998). Bacteria present in the middle ear or refluxed from the nasopharynx may cause a secondary infection and prolong the inflammatory response, leading to a chronic disease state.
2. A first reflux episode from the nasopharynx would cause inflammation and the secretion of neutralising substances such as mucus and HCO_3^- but not enough to cause ET obstruction. Some pepsin may not be completely denatured, remaining inactive at the high pH and if subsequent reflux events occur the pepsin may be reactivated causing further damage and inflammation.

Exposure of the ET and middle ear to acid reflux would cause direct tissue damage, leading to inflammation, oedema (Cherry and Margulies, 1968) and ciliostasis (Holma *et al.*, 1977). Ciliostasis of the ET mucosa would disable the mucociliary clearance

mechanism, potentiating the formation of a middle ear effusion (Ohashi *et al.*, 1986; Park *et al.*, 1993).

1.3.5 Gastric juice composition

Gastric juice is a fluid secretion containing hydrochloric acid (pH0.9-1.5) and a group of proteolytic enzymes, pepsins. Hydrochloric acid is secreted by parietal cells that are mainly present in the fundus and body regions of gastric mucosa. Pepsins are secreted extracellularly by chief cells in a precursor form, pepsinogen, which is rapidly converted to an active proteinase under acidic conditions (Samloff, 1971). Pepsin and acid are the natural aggressors present in gastric juice.

Two immunological groups of pepsinogens have been isolated with different tissue origins, pepsinogen I (PGI), secreted by the oxyntic glands of the fundic mucosa and pepsinogen II (PGII), secreted by the pyloric glands of the antrum, duodenum and oxyntic glands (Allen *et al.*, 1989). PGI contains pepsinogens 1–5 and PGII contains pepsinogens 6 and 7. The proenzyme pepsinogen is converted to pepsin in the gastric gland under acidic conditions. It is a multistage process that is spontaneous at pH<4.5. It is presumed that each pepsinogen gives rise to a specific pepsin (Hirschowitz, 1984), with pepsins 1, 2, 3a, 3b, 3c and 4 (pepsins A) corresponding to PGI whilst pepsins 5 and 6 (pepsins C) belong to PGII.

Pepsinogens have been isolated and sequenced and complete amino acid sequences have been determined for many species including human, pig and chicken (Baudys and Kostka, 1983; Sogawa *et al.*, 1983; Lin *et al.*, 1989). Pepsinogens consist of a single polypeptide chain of approximately 370 amino acids e.g. porcine pepsinogen A is 371 amino acids in length (Hartsuck *et al.*, 1977). The overall structures of porcine

pepsinogen A, human pepsinogen A (of which the major pepsin is pepsin 3) and human progastricsin (the precursor of pepsin 5) are alike, however the similarity between porcine and human pepsinogens is greater than between human pepsinogen A and human progastricsin (Richter *et al.*, 1998).

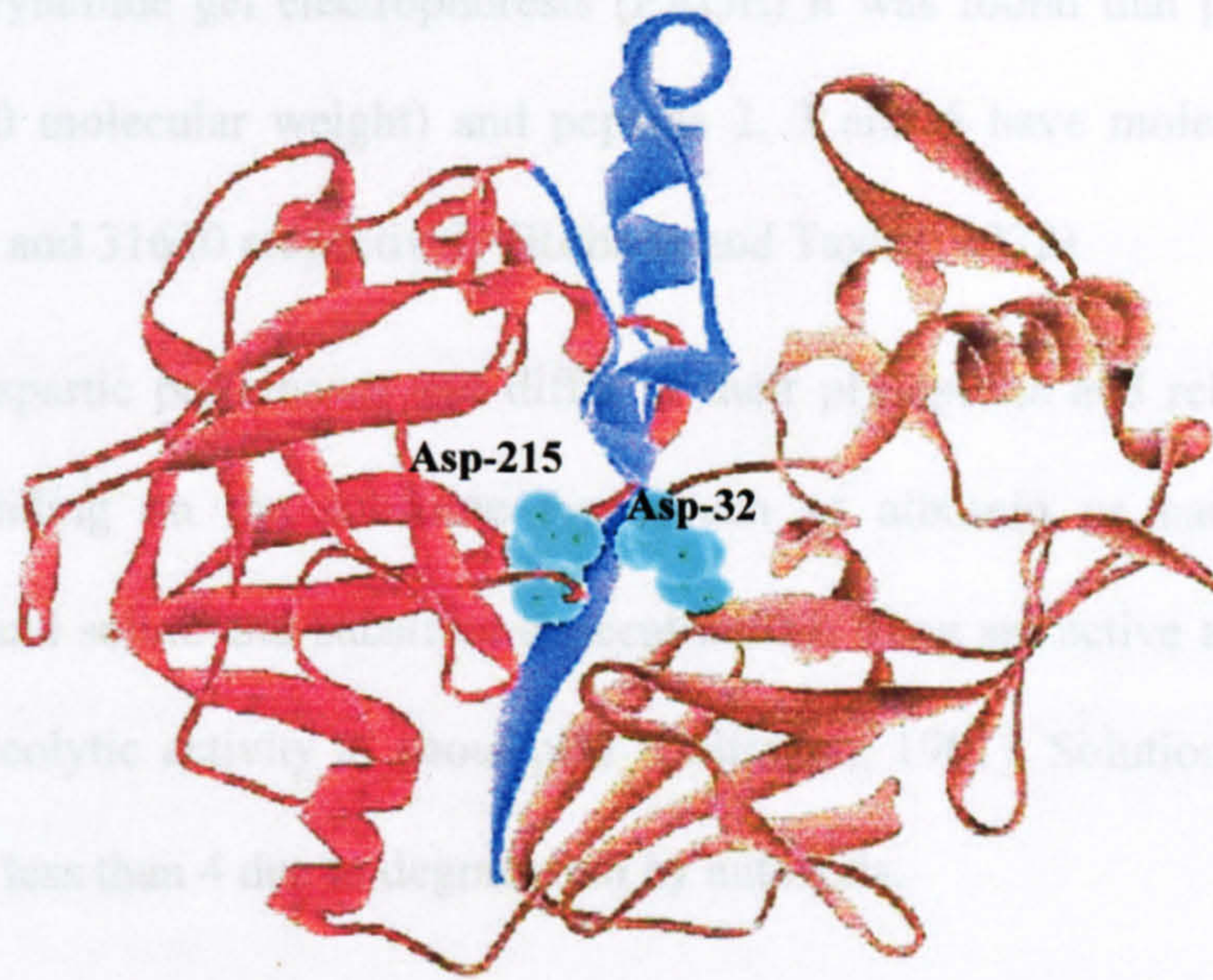
Native pepsinogen contains a prosegment and a pepsin moiety. The pepsin moiety consists of two domains, related by a two-fold axis of symmetry. At the junction of the two domains is an extended substrate binding cleft which contains two catalytically important aspartate residues Asp-32 and Asp-215 (James and Sielecki, 1986) (Figure 1.10). This binding cleft is covered and occupied by the prosegment, which is bound to the pepsin moiety mainly through electrostatic interactions. The active site is fully formed in the proenzyme therefore the prosegment serves to block entry of the substrate to the active site and is crucial in maintaining the molecule in its inactive form at neutral pH.

The prosegment is present at the N-terminus of the molecule and contains relatively high amounts of basic residues whereas the pepsin moiety is characteristically highly acidic (Kageyama, 2002). The following is the activation sequence for porcine pepsinogen A:

At neutral pH, the carboxyls of the pepsin moiety are negatively charged and the positive charge of the prosegment is essential to stabilise the pepsinogen molecule in its inactive conformation (Andreeva and James, 1991). At pH<4.5 the carboxyls of the pepsin moiety become protonated, disrupting the electrostatic interactions between the prosegment and the active enzyme, allowing the prosegment to undergo the conformational changes that initiate the activation reaction. The active site is then exposed and the pepsinogen molecule cleaves off its own prosegment autocatalytically

via a direct or a sequential pathway. The prosegment is 44 amino acids long in the pig (Foltmann, 1981) and 47 amino acids long in the human (Kageyama and Takahashi, 1980) and activation occurs by a similar mechanism. After cleavage of the prosegment there are two further conformational changes, the dissociation of the prosegment from the active site and then a change in the conformation of the first thirteen residues at the N-terminus of the active enzyme from a random-coil to a β sheet (McCaman and Cummings, 1988), resulting in an active pepsin molecule whose secondary structure consists almost entirely of β sheets (Konno *et al.*, 2000). It has not been possible to isolate the intact 44 amino acid prosegment peptide after cleavage from the active porcine pepsin molecule, instead several shorter peptides (corresponding to peptides 1-16, 17-24, 25-44, 17-44 and 44-45) can be isolated (Dunn *et al.*, 1978), resulting from multiple proteolytic cleavages (James and Sielecki, 1986) of which peptide 1-16 is pepstatin. This peptide was found to interact and strongly inhibit the activity of pepsin at pH5.5 (Dunn *et al.*, 1978).

Figure 1.10 Tertiary structure of porcine pepsinogen A (Richter *et al.*, 1998)



The active enzyme moiety is shown in red, consisting of two domains. At the junction of the two domains is the extended substrate-binding cleft, containing the two active site aspartates (Asp-32 and Asp-215) shown in blue. The binding cleft is covered and occupied by the prosegment, shown in purple.

There are seven human pepsins; pepsin 3 is always the most abundant of pepsins present in gastric juice, followed by pepsin 5. The other pepsins are present in much smaller quantities except for pepsin 1, which makes a variable contribution to peptic activity. Using polyacrylamide gel electrophoresis (PAGE) it was found that pepsin 1 was the largest (43810 molecular weight) and pepsins 2, 3 and 5 have molecular weights of 39950, 37150 and 31620 respectively (Roberts and Taylor, 1972).

Pepsins are aspartic proteinases and differ in their pH optima and relative proteolytic activity depending on the substrate type (such as albumin or haemoglobin), pH, temperature, and solute and substrate concentrations. They are active at acidic pH with optimum proteolytic activity at about pH2 (Foltmann, 1981). Solutions of pepsin lose activity at pH less than 4 due to degradation by autolysis.

Pepsins are liable to irreversible denaturation at $\text{pH} > 7.2$ (Piper and Fenton, 1965; Hirschowitz, 1984), whereas pepsinogens are not denatured by pH as high as pH10. Pepsins are enzymatically inactive at pH6 but are stable and will become active when the pH is lowered towards their pH optima (Piper and Fenton, 1965). In neutral or alkaline solution, pepsin is known to undergo a conformational transition to an irreversibly denatured state that is partially structured rather than fully unfolded (Konno *et al.*, 2000). Energy calculations have predicted that the C-terminal lobe is more stable at high pH than the N-terminal lobe and it has been demonstrated that alkaline inactivation of pepsin is due to a selective denaturation of its N-terminal lobe (Lin *et al.*, 1993).

1.3.6 Treatment in children

There are a variety of approaches of treatment of gastro-oesophageal reflux and laryngopharyngeal reflux and they fall into three general categories (Andze *et al.*, 1991; Smit *et al.*, 2000; Zalzal and Tran, 2000; Carroll *et al.*, 2002):

Non-pharmacological	Pharmacological	Surgical
Head elevation	Antacids	If severe complications are suffered, fundoplication may be considered, a procedure where the fundus is wrapped around the lower oesophagus, reinforcing the LOS and preventing gastric contents from refluxing into the oesophagus as easily
Milk thickening	H ₂ antagonists	
Positioning changes	Prokinetic agents (stimulate stomach emptying)	
Reduced pacifier use	Proton pump blockers	
Avoidance of substances that may decrease LOS tone such as chocolate, coke, cacao, mint or peppermint		

Treatment depends on age, severity of symptoms, oesophagitis and outcome of initial treatment. The first stage is generally a modification in lifestyle and is non-pharmacological and there is a lack of evidence for the success of these therapies (Carroll *et al.*, 2002). If symptoms of reflux persist then drug treatment may be necessary. Medical treatment (i.e. not requiring surgery) is successful in over 80% of cases (Andze *et al.*, 1991).

CHAPTER 2

MATERIALS & METHODS

Chapter 2 Materials and Methods

2.1 Reagents

Unless otherwise indicated all chemicals were obtained from BDH Merck Ltd., Fisher Scientific UK or the SIGMA-Aldrich Company Ltd. and were of AnalaR grade where available.

2.2 Source and collection of samples

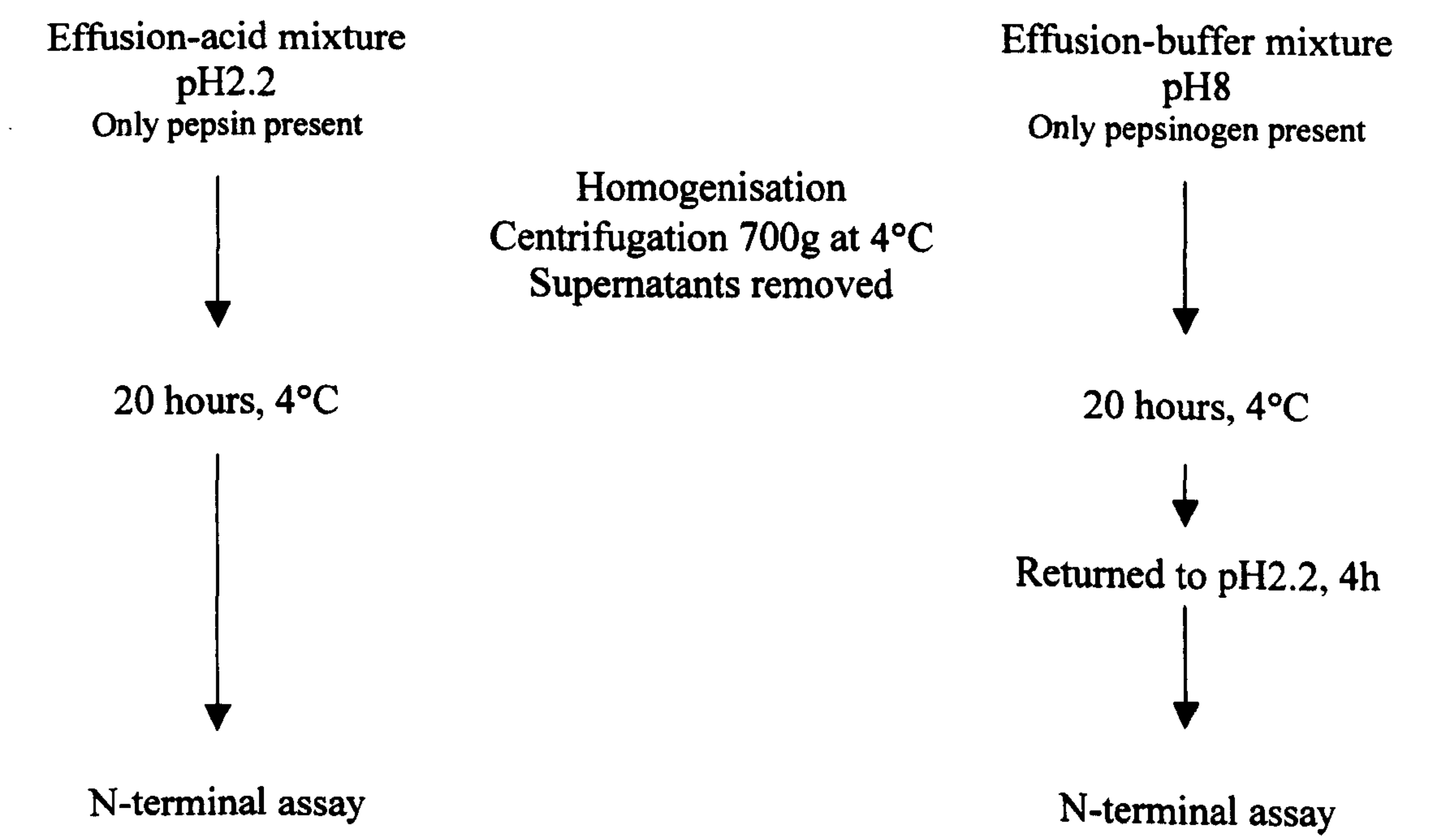
Middle ear effusions were collected by Professor John Birchall at Queens Medical Centre, Nottingham and by Mr Sean Carrie at the Freeman Hospital, Newcastle upon Tyne. The samples were obtained from children aged between two and eight years undergoing myringotomy after diagnosis of persistent otitis media with effusion (with the presence of an effusion for three months or more). Effusions were removed from the middle ear cavity by suction supplied by a vacuum line with a specially designed instrument and collected into small containers. The effusions were immediately frozen and stored at -20°C until required.

Samples were categorised by the clinician into two groups by visual inspection. Viscous (mucoid) effusions that did not flow upon inversion of the container were classified as 'thick effusions'. Samples that did flow (serous) were classified as 'thin effusions'. Effusion colour varied from a pale straw yellow through to dark yellow, brown and deep red. Some thin effusions were colourless and translucent. Both types of sample varied in volume from 20-400µl. Effusion samples were individually analysed unless otherwise stated.

2.3 Preparation of effusion samples

2.3.1 Proteinase activity studies

Middle ear effusion samples were thawed at 4°C and added to a known volume of 0.01M HCl (pH2.2) or 0.067M phosphate buffer (pH8). The effusion-acid and effusion-buffer (alkali) mixtures were homogenised using a hand-held Griffiths tube homogeniser until a consistent homogenate was produced (2-3 minutes). The homogenates were centrifuged for 1 hour at 700g and 4°C to remove insoluble material from the effusions. The resulting supernatants were removed and stored overnight (20 hours) at the respective pHs. The pH8 samples were returned to pH2.2 by dialysis for 4 hours in 0.01M HCl before analysis of proteolytic activity in the samples was carried out. See diagram below.



2.3.2 Mucin and cytokine studies

Effusion samples were thawed at 4°C and washed out of the collection containers using a known volume of ice-cold 0.067M phosphate buffer, pH6.5 containing a cocktail of proteolytic inhibitors to prevent degradation by endogenous proteolytic enzymes. The protease inhibitors included are shown in Table 2.1. The resulting effusion-buffer mixtures were homogenised as previously described using a hand-held Griffiths tube homogeniser to obtain a consistent homogenate.

Table 2.1 Concentration and action of protease inhibitors within the proteolytic inhibitor cocktail

Protease inhibitor	Concentration (mM)	Mode of action
Phenylmethyl-sulphonyl fluoride (PMSF)	3	Inhibitor of serine proteases, e.g. elastase (hydrolyses peptide bond at C-terminal side) and some cathepsins and trypsin (remove N-terminal dipeptides)
Sodium iodoacetate	1	Inhibitor of thiol-dependent proteases, e.g. cathepsin B and other thiol-dependent enzymes
Benzamidine Hydrochloride	15	Specific inhibitor of trypsin and trypsin-like serine proteases (hydrolyse proteins, peptides and amino acids)
Na ₂ EDTA	10	Inhibitor of metallo-dependent proteases, e.g. Ca ²⁺ dependent enzymes, collagenases
N-ethylmaleimide	10	Prevents disulphide group exchange
α-amino caproic acid	100	Inhibitor of plasminogen activation to serine protease plasmin (hydrolyses peptide and ester bonds)

2.4 Analytical Methods

2.4.1 N-terminal assay

Proteolytic activity, measured at pH2.2 was determined by the N-terminal assay of Hutton (Hutton *et al.*, 1986), a modification of Lin (Lin *et al.*, 1969) using succinyl albumin as the substrate for proteolytic cleavage. Bovine serum albumin was succinylated by the method of Furihata (Furihata *et al.*, 1978). The N-terminal assay directly assesses the ability of any pepsin present to hydrolyse peptide bonds. These newly liberated N-terminals can then be tri-nitrophenylated and quantified colorimetrically.

Pepsin levels (μgml^{-1}) were measured by its ability to hydrolyse peptide bonds at pH2.2 using succinyl albumin as substrate. Standard curves were prepared using porcine pepsin A, 0-1 μg in 200 μl 0.01M HCl (pH2.2). Samples and substrate were prepared in 0.01M HCl. All other reagents were prepared in distilled water.

Enzyme (0.2ml, 0.1-1 μg pepsin standard and known sample dilutions) were mixed with 0.5ml of substrate (8 mgml^{-1} succinyl albumin in 0.01M HCl, pH2.2) to start the reaction. Samples were mixed and incubated for 30 minutes at 37°C. The reaction was stopped by the addition of 4% (w/v) sodium bicarbonate (0.5ml) followed by 0.05% (w/v) trinitrobenzene sulphonic acid (TNBS, 0.5ml). Colour was developed by incubating at 50°C for 10 minutes followed by addition of 0.5ml of 10% sodium dodecyl sulphate (SDS) and 0.25ml of 1M HCl to complete the reaction and stop further colour development. Absorbance was measured at 340nm.

Time zero controls were prepared by addition of substrate at the end of the 30 minutes incubation, just before addition of sodium bicarbonate. This gave a measure of all free N-terminals existing before hydrolysis on both enzyme and substrate.

The standard curve using porcine pepsin was only linear between concentrations of 0 to 1µg enzyme in the total reaction volume (0.7ml) and therefore this range of concentration was used to calculate pepsin activity in the samples. The optimum activity of pepsin is at pH2.2 (Roberts and Taylor, 1972) therefore the N-terminal assay is carried out at this pH to ensure consistency.

2.4.2 Slot Blot / Enzyme Linked Immunosorbent Assay

Nitrocellulose membrane (pore size 0.45µm or 0.2µm) supported on blotting paper was wetted and inserted into a minifold 72 well slot blot apparatus (Schleicher and Schuell) and connected to a vacuum source. Standards and samples were added to individual wells and allowed to absorb onto the membrane under vacuum. The nitrocellulose membrane was removed from the apparatus and placed into a container and an enzyme linked immunosorbent assay (ELISA) performed. To block non-specific binding, the membrane was incubated in phosphate buffered saline (PBS) containing 2% bovine serum albumin (BSA) overnight at 4°C. The membrane was then incubated in primary antibody diluted in 1% BSA/PBS at room temperature for 1 hour. Unbound antibody was removed by rinsing in 2 changes of 0.5% TWEEN 20/PBS followed by 3 changes of PBS. Horseradish peroxidase (HRP) conjugated secondary antibody diluted in 1% BSA/PBS was added and incubated for 1 hour. After rinsing as described previously, colour was developed with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB), 0.03% hydrogen peroxide (H₂O₂) in PBS for 5 minutes. After rinsing in tap water the

membrane was air-dried and absorbance measured at 595nm using a Shimadzu scanning densitometer.

2.4.2.1 Antibodies

Unless otherwise explained, all antibody dilutions used had been predetermined either by the manufacturer or previous research.

Purified mucin from thick effusions of anatomically normal children was used to prepare polyclonal antiserum (TEPA) in rabbits. This antiserum reacts strongly with human salivary mucin (of which MUC5B is the major fraction) (Hutton *et al.*, 1998a). The specificity of the TEPA antiserum had previously been determined by immunohistochemical and papain digestion studies (Hutton *et al.*, 1998a). The pattern of staining with the TEPA antibody (Hutton *et al.*, 1998b) showed negative results in stomach and ileum tissues, positive results in salivary glands and strong staining of the mucous glands of the middle ear mucosa. No staining was observed in non mucus secreting cells and tissues. Papain digestion studies demonstrated that the antibody was not recognising the major carbohydrate-containing domain or any epitopes on that domain. TEPA almost certainly recognises a protein epitope in the non-tandem repeat region (Hutton *et al.*, 1998a). This antibody was used at a dilution of 1:10000.

Mouse anti-human gastric mucin MUC5AC antibody, NCL-HGM-45M1, was obtained from Novocastra Laboratories Ltd. Newcastle. This monoclonal IgG1 antibody was raised to mucin isolated from ovarian cyst fluid and recognised a mucin epitope located in the non-glycosylated region of the gastric mucin MUC5AC peptide core. The antibody was used at a dilution of 1:200.

Rabbit anti-human MUC7 antibody was made at Newcastle University molecular biology unit. The polyclonal antiserum was raised to a synthetic peptide with the amino acid sequence NLLNRIIDDMVEQ found in a unique region of the C-terminal domain of the human MUC7 gene product. This sera was used at a dilution of 1:1000.

Goat anti-pepsin (porcine stomach) antibody was obtained from Biodesign International, Maine, USA. This was an IgG fraction obtained from monospecific antiserum by a multistep process including delipidation, salt fractionation and ion-exchange chromatography. The antibody was used at a dilution of 1:2000.

Rabbit anti-human fibrinogen antibody was obtained from DAKO, Copenhagen, Denmark. This polyclonal antiserum was raised to fibrinogen isolated from human plasma and was unable to distinguish between fibrin and fibrinogen. The sera was used at a dilution of 1:2000.

Secondary antibodies were obtained from SIGMA-Aldrich. These were goat anti-rabbit IgG (whole molecule) peroxidase conjugate used at a dilution of 1:10000, goat anti-mouse IgG (whole molecule) peroxidase conjugate used at a dilution of 1:5000 and mouse anti-goat IgG peroxidase conjugate used at a dilution of 1:10000.

2.4.3 Solid Phase Sandwich ELISA

A quantitative sandwich enzyme immunoassay technique (Voller *et al.*, 1978) was used for the determination of human interleukin-6 and interleukin-8 cytokines in middle ear effusions.

2.4.3.1 *Interleukin-6 (IL-6)*

A Human IL-6 ELISA OPT-EIA™ Kit was obtained from BD Biosciences, San Diego, USA. Standards and samples were added in duplicate to microplate wells pre-coated with anti-human IL-6 monoclonal antibody and incubated at room temperature for two hours. Unbound material was removed by washing the wells five times with a detergent solution (PBS with Tween-20 and thimerosal as a preservative). Avidin-horseradish peroxidase conjugate was mixed with biotinylated anti-human IL-6 monoclonal antibody and added to the wells to produce an antibody-antigen-antibody “sandwich”. The plate was incubated for one hour at room temperature then the wells were washed seven times with detergent solution. TMB substrate solution (3,3', 5,5'-tetramethylbenzidine) was added to each well and the plate was incubated for 30 minutes at room temperature in the dark. The TMB substrate produced a blue colour in direct proportion to the amount of IL-6 present in the initial sample. After 30 minutes the stop solution containing 1M phosphoric acid was added to the wells. This changed the colour from blue to yellow and the microwell absorbances were measured at 450nm using a Multiskan Ascent Microplate photometer (Thermo Labsystems Ltd., UK).

2.4.3.2 *Interleukin-8 (IL-8)*

A Quantiglo Human IL-8 Chemiluminescent Immunoassay was obtained from R&D Systems, Abingdon, Oxon. Standards and samples were added in duplicate to microplate wells precoated with a murine monoclonal antibody against IL-8 and incubated for two hours at room temperature. Any unbound material was removed by washing the wells four times with a wash buffer (buffered surfactant with preservative). Polyclonal antibody against IL-8 conjugated to horseradish peroxidase was added to the wells and the plate was incubated for three hours at room temperature. The wells were washed as above. The immunoassay development and quantification step was based on a light emitting substrate. For the purposes of this assay this substrate was replaced by a peroxidase substrate 2,2'-azino-bis(ethylbenzthiazoline-6-sulfonic acid) which was added to the wells for 30 minutes and the reaction stopped by the addition of an equal volume of 1% SDS. A blue-green soluble reaction product developed that was directly proportional to the amount of IL-8 present in the sample and the absorbances were measured at 405nm using a Multiskan Ascent Microplate photometer (Thermo Labsystems Ltd., UK).

2.4.4 Glycoprotein Assay

Glycoprotein was quantified using the periodic acid-Schiff's (PAS) assay (Mantle and Allen, 1978). Standard curves were prepared using 0-150 μ l of 1mgml⁻¹ pig gastric mucin (SIGMA-Aldrich) (that was subsequently papain digested) and made up to a total volume of 1ml of distilled water. 200 μ l of periodic acid solution (0.2% (v/v) periodic acid (BDH) in 7% acetic acid) was added to 1ml of standard or sample and incubated at 37°C for 1 hour. Schiff's solution (SIGMA-Aldrich) (0.017% (w/v) sodium

metabisulphite (BDH) in Schiff's reagent (fuschin-sulphite reagent)) was pre-incubated at 37°C for 1 hour. 200µl of Schiff's solution was added to each tube and left for 30 minutes at room temperature to develop colour. Absorbance was measured at 555nm against the reagent blank and the amount of glycoprotein in the sample determined from the standard curve.

2.4.5 Protein assay (Bradford)

Protein was estimated using the Bio-Rad assay kit (Bradford, 1976) (Bio-Rad Laboratories Ltd, UK). Standard curves were prepared using bovine serum albumin to give a range between 0 and 20µg, in a total volume of 0.8ml distilled water. Samples were diluted as necessary to a volume of 0.8ml. 0.2ml Coomassie blue reagent (BioRad reagent) was added to each sample and standard. After incubation at room temperature for 30 minutes, the absorbance was measured at 595nm and the sample protein content determined from the standard curve.

2.4.6 Protein and nucleic acid estimation

Protein and nucleic acid content were estimated by measurement of absorbance at 280nm and 260nm. The protein and nucleic acid content of samples was extrapolated from a nomograph based on the extinction coefficients for enolase and DNA (Warburg and Christian, 1942)

2.5 Mucin Studies

2.5.1 Dilute solution viscosity studies

Measurements were made using a Couette rotating cup viscometer (Contraves low shear 30) controlled by an electronic speed programmer (Rheoscan 20), which allowed the speed of the cup to be increased under controlled conditions. The temperature of the apparatus was maintained by a thermostatically controlled water bath circulating water through the viscometer block. The speed (shear rate) programme produced a range of up to 30 fixed speeds in a geometric progression or generated continuously variable speeds throughout the same range.

Effusions were homogenised in 0.067M phosphate buffer, pH6.5 containing a proteolytic inhibitor cocktail. 2ml of the homogenate was placed in the cup, and the bob, suspended by a torsion wire, was lowered into the sample giving a 0.1mm annulus of sample between the cup and bob. Viscosity measurements were made at 37°C in the rotating cup between speeds of 1.7s^{-1} and 128.5s^{-1} . Before any measurements were made, samples were sheared twice, through and back through the whole shear range to disrupt any weak non-specific non-covalent interactions. Speed versus angular deflection (in degrees) plots were obtained (% deflection being plotted on the Y axis and speed on the X axis of an X.Y. recorder), the gradients of which are directly proportional to the viscosity of the solution under test. Gradients were also produced for buffer alone and the ratio of these sample gradients to buffer gradient calculated to be the relative viscosity (η_{rel}).

Relative viscosity (η_{rel}). = % deflection of test solution / % deflection of buffer

Specific viscosity (η_{sp}). = Relative viscosity – 1

2.5.2 Dialysis and freeze drying

After effusion viscosity had been measured, samples were placed into visking dialysis tubing (Medicell International Ltd., UK). Exhaustive dialysis of samples was achieved in 20 litres of distilled water at 4°C with continuous stirring for at least three days with six changes of water daily. Dialysis tubing had a molecular weight cut off point of 12–15000Da. The samples were rinsed out of the dialysis tubing using distilled water, frozen at -80°C for one hour and freeze dried on a Modulyo freeze drier attached to a Pirani 10 vacuum pump and subsequently stored at -20°C.

2.5.3 Equilibrium Density Gradient Centrifugation

To purify effusion material (or saliva samples) by removal of protein and nucleic acid, equilibrium density gradient centrifugation in caesium chloride (CsCl) was used (Starkey *et al.*, 1974). CsCl was added to the samples in solution to give a starting density of 1.42gml⁻¹ and the solution centrifuged at 100,000g for 48hours at 4°C, using a fixed angle rotor.

Without disturbing the resulting gradient, the content of each tube was separated into nine equal fractions. The density of each fraction was measured, followed by exhaustive dialysis against distilled water at 4°C. Dialysed fractions were assayed for glycoprotein and protein and an estimation of nucleic acid was made. Fractions rich in protein and nucleic acid were generally discarded. Fractions rich in glycoprotein were pooled, freeze dried and stored at -20°C.

2.6 Size Fractionation Methods

2.6.1 Gel Filtration

Gel filtration was used to attempt to separate high molecular weight purified salivary mucin (MUC5B) from lower molecular weight mucin (containing MUC7) and low molecular weight protein contaminants. A Sepharose CL-4B column (1.5 x 30cm) and a Sepharose CL-2B column (1.5 x 130cm) were prepared as described in “Gel Filtration: Principles and Methods” published by Amersham Biosciences UK Ltd. (2000).

The void volume (V_o) and total volume (V_T) of columns were determined by fractionating a solution of 0.5% (w/v) blue dextran containing 0.05% (w/v) methyl orange. Both columns were eluted with 0.2M sodium chloride containing 0.02% (w/v) sodium azide. The Sepharose CL-4B column was eluted by downward flow under gravity and the CL-2B column was eluted by upward flow with the aid of a peristaltic pump. Freeze dried CsCl purified mucin was reconstituted at concentrations of $1\text{--}3\text{mgml}^{-1}$ in 0.2M NaCl/0.02% NaN_3 and 1 or 2ml volumes were loaded on the column. 1 or 3ml fractions were collected and assayed for glycoprotein. Excluded fractions which eluted at V_o , were pooled as the respective high molecular weight mucin (MUC5B). Glycoprotein-rich fractions that were in the included volume were pooled and assayed for the presence of MUC7 by ELISA.

2.6.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)

SDS PAGE was conducted using the Pharmacia PHAST gel system (Pharmacia Ltd.). Freeze dried samples or standards were solubilised in either 0.0625M Tris buffer, pH 6.8, containing 2% (w/v) SDS, 10% (v/v) glycerol, 0.001% bromophenol blue (non-

reducing buffer), or 0.0625M Tris buffer, pH 6.8, containing 2% (w/v) SDS, 10% (v/v) glycerol, 0.001% bromophenol blue and 5% mercaptoethanol (reducing buffer).

Samples were heated at 100°C for two minutes in a boiling waterbath before being applied to the gels (Phastgel™ Gradient 4-15% or 8-25%). Gels were run at constant current of 100mA for 260Vh until the tracking dye had reached the end of the running gel. Gels were stained for glycoprotein using the PAS method (Van Seuningen *et al.*, 1992) (Table 2.2). The gels were then scanned for glycoprotein at 555nm using a Shimadzu scanning densitometer.

The amount of polymeric material present in a sample was calculated by determining the amount of material that remained at the point of application compared to the amount that had moved from the point of application to the interface between the stacking and running gel and into the running gel. This SDS-PAGE method has previously been validated (Rankin *et al.*, 1995; Newton, 1998).

Purity of mucin (detection of protein contaminants) was assessed using SDS PAGE and staining gels overnight in 0.05% (w/v) coomassie blue, 25% (v/v) propan-2-ol and 10% (v/v) acetic acid. Gels were then destained for one day in 25% (v/v) propan-2-ol and 10% (v/v) acetic acid followed with overnight destaining with 10% (v/v) propan-2-ol and 10% (v/v) acetic acid. Gels were then scanned for protein at 585nm using a Shimadzu scanning densitometer.

Table 2.2 Method of staining gels for glycoprotein using the PAS method

Step		Time (min)	Temp (°C)
1	20% trichloroacetic acid	5	20
2	deionised water	5	20
3	0.7% periodic acid + 5% acetic acid	10	20
4	deionised water	2	20
5	deionised water	2	20
6	Schiff's reagent	10	20
7	5% potassium disulphite + 5% acetic acid	5	20
8	5% methanol + 7.5% acetic acid	10	50
9	5% methanol + 7.5% acetic acid	5	50
10	50% methanol + 30% acetic acid	10	50
11	50% methanol + 30% acetic acid	5	50
12	50% methanol + 30% acetic acid	2	50
13	5% glycerol + 10% acetic acid	10	50

(From Van Seuningen & Davril, 1992)

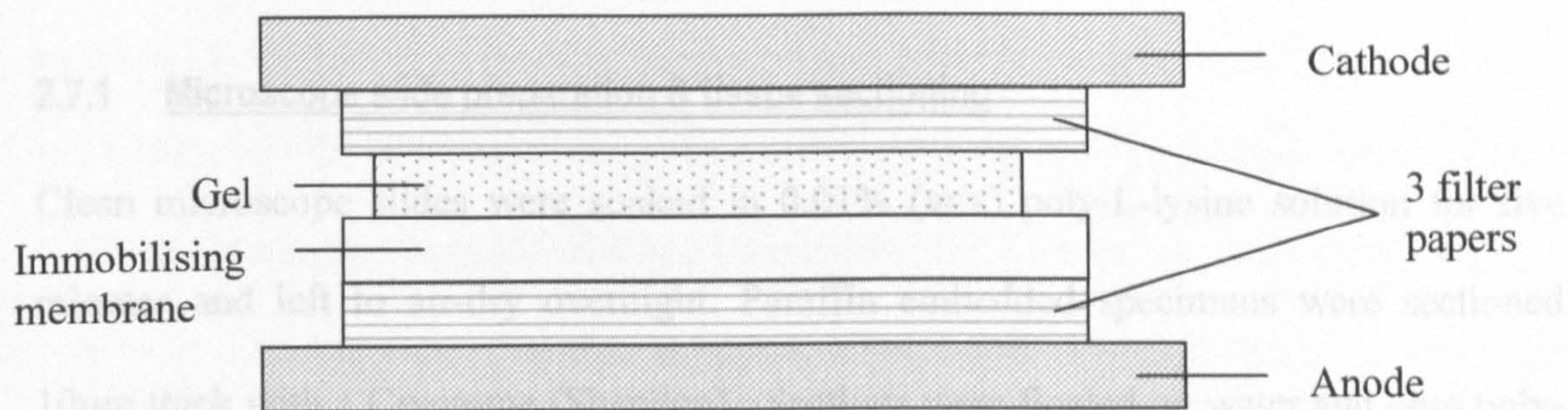
2.6.3 Immunoblot Procedure (Western blotting)

Western blotting was used to detect proteins present in samples using their molecular weight and antigenic properties.

After separation of the sample / standard by SDS PAGE, proteins were transferred from the polyacrylamide gels to nitrocellulose membranes using a PhastTransfer Semi-dry Electrophoretic Transfer System (Pharmacia Ltd.) within the PhastSystem. This process was undertaken immediately after the electrophoresis run as proteins begin diffusing within the gel as soon as the run is completed.

The polyacrylamide gel was separated from its backing (but not fully detached) and a nitrocellulose membrane (0.2µm pore size) pre-wetted with transfer buffer (25mM Tris, 192mM glycine, 20% methanol and pH8.3) was placed on the surface of the gel. The gel assembly was turned over and the membrane peeled from the gel backing. A “transfer sandwich” was made in the Electrode Cassette, which incorporated the electrodes required for protein transfer. The sandwich consisted of three pre-wetted filter papers (in transfer buffer), the gel with attached membrane so that the membrane was in direct contact with the filter paper and then three more pre-wetted filter papers on top of the gel (see diagram below). The cathode was placed on top of the filter papers and the Electrode Cassette was loaded into the separation compartment of the PhastSystem. This was now ready for protein transfer.

The complete transfer sandwich



The conditions used for the protein transfer were as follows:

Voltage	20V
Current	25mA
Power	1W
Temperature	15°C
Volthours	5Vh

After protein transfer was achieved, the transfer sandwich was removed and the membrane peeled away for further identification of protein bands. It was not possible to stain the gel to check for transfer as its backing had been removed. The membrane was incubated in phosphate buffered saline (PBS) containing 2% bovine serum albumin (BSA) overnight at 4°C to block non-specific binding and then probed with antibodies using an ELISA technique (Section 2.4.2).

2.7 Histological Methods

2.7.1 Microscope slide preparation & tissue sectioning

Clean microscope slides were soaked in 0.01% (w/v) poly-L-lysine solution for five minutes and left to air-dry overnight. Paraffin embedded specimens were sectioned 10µm thick with a Cryotome (Shandon). Sections were floated on water and onto poly-L-lysine coated slides and air-dried.

2.7.2 Haematoxylin and Eosin Staining

To observe general histological architecture of tissue sections, slides were stained with haematoxylin and eosin (H & E). After dewaxing, slides were fixed in 10% neutral buffered formalin (20 seconds) then briefly immersed in acetic acid/ethanol (25% (v/v) glacial acetic acid in absolute ethanol). The slides were transferred into 70% ethanol for 20 seconds. Slides were then rinsed under slowly running tap water to remove any solvent residue and then immersed in Gill No. 2 haematoxylin (Sigma) for two minutes. Slides were then rinsed to remove excess dye followed by a rinse in ethanol 70%. They were then immersed in Blueing solution (ammoniated alcohol) until they took on a blue colour, rinsed and stained in aqueous Eosin Y solution (1%) (BDH). After rinsing well in water the slides were dehydrated and mounted with a coverslip using DPX mountant.

2.7.3 Immunohistochemistry

To determine the presence or absence of antigens in middle ear mucosa biopsy tissue, immunohistochemistry (IHC) was employed. The slides were de-waxed and rehydrated to PBS. Microwave antigen retrieval was performed in citrate buffer (0.8mM citric acid,

82mM tri-sodium citrate, pH6) then slides were rinsed in PBS. Endogenous peroxidase activity was blocked by incubating the slides in 1% (v/v) H₂O₂ in methanol for 20 minutes. After rinsing in running tap water, non-specific binding was blocked with 3% BSA/PBS for 20 minutes. Slides were drained and then incubated with primary antibody diluted in 1% BSA/PBS in a humid chamber for one hour. Unbound antibody was removed by rinsing slides in three changes of 1% BSA/PBS. Horseradish peroxidase-conjugated secondary antibody, diluted in 1% BSA/PBS was added and incubated in a humid chamber for 30 minutes. After rinsing as described previously, colour was developed with 0.05% 3'3 diaminobenzidine tetrahydrochloride (DAB)/0.03% H₂O₂ in PBS for ten minutes. Slides were rinsed, counterstained in haematoxylin for two seconds, and rinsed well in running tap water, then dehydrated in graded alcohols to histoclear and mounted in a xylene based mountant.

For details of antibodies used in this procedure see section 2.4.2.1.

2.8 Statistical Analyses

Data were expressed as mean \pm 1 SEM unless otherwise stated in the text. Data were analysed by unpaired Student's t-test and were considered significant when $P < 0.05$.

CHAPTER 3

GASTRO-OESOPHAGEAL REFLUX AND OME

Chapter 3 Gastro-oesophageal reflux and OME

3.1 Introduction

Paediatric gastro-oesophageal reflux (GOR) has been frequently reported (Vandenplas *et al.*, 1988) and linked to the development of many airway disorders such as recurrent pneumonia, chronic asthma, rhinitis and sinusitis, recurrent croup, stridor and laryngitis (Berquist *et al.*, 1981; Koufman, 1991). The presence of acid reflux in the nasopharynx, detected by nasopharyngeal pH monitoring, has been documented (Contencin and Narcy, 1991) and it has been postulated that GOR may be involved in the pathogenesis of an entire spectrum of otorhinolaryngologic conditions (Contencin *et al.*, 1995; Yellon, 1997) including otitis media with effusion (Shilkin, 1994; Halstead, 1999; Velepich *et al.*, 2000).

It is thought that the aetiology of OME is related in part to the anatomic and immunologic immaturity of the paediatric Eustachian tube (ET) (Bluestone, 1996; Sudo and Sando, 1996) and that ET dysfunction is an important factor in the pathogenesis of the disease (Bylander-Groth and Stenstrom, 1998). The ET in children is shorter and less inclined (13–18mm and 10°) (Proctor, 1967) from the tympanic orifice to the pharyngeal orifice than the mature ET in adults (31–38mm and 45°) (Sadler-Kimes *et al.*, 1989). These anatomic differences are believed to impair the protective function of the ET and this combined with the fact that infants and neonates are often placed in the supine position, may allow gastric contents to travel from the nasopharynx into the middle ear cleft. Any acid reflux into the middle ear may alter the local immune system

and cause inflammation in the pharynx and ET as acidity and acidic proteinase activity in these areas is abnormal (Holma *et al.*, 1977; Contencin and Narcy, 1991).

Many researchers have suggested the link between GOR and OME, however a direct relationship has not yet been determined. It has been demonstrated that repeated exposure of the rat middle ear to simulated gastric refluxant causes worsening ET function with time (Heavner *et al.*, 2001a; Heavner *et al.*, 2001b). Cures or reduction in the frequency of recurrent otitis media with anti-GOR treatment have been reported (Bouchard *et al.*, 1999; Poelmans *et al.*, 2001). In one study, six children were referred for recurring otitis media, these children either had oesophagitis or abnormal pH monitoring, after treatment of GOR, four of the patients had complete resolution of their symptoms and two were significantly improved (Contencin *et al.*, 1995). The role of GOR in OME needs to be further investigated and evidence for gastric juice in the middle ear sought. As gastric juice contains pepsins, this can be done by analysis of middle ear effusions for the presence of acidic proteinase (pepsin) activity and pepsin protein.

3.2 Analysis of middle ear effusions for the presence of acidic proteinase activity

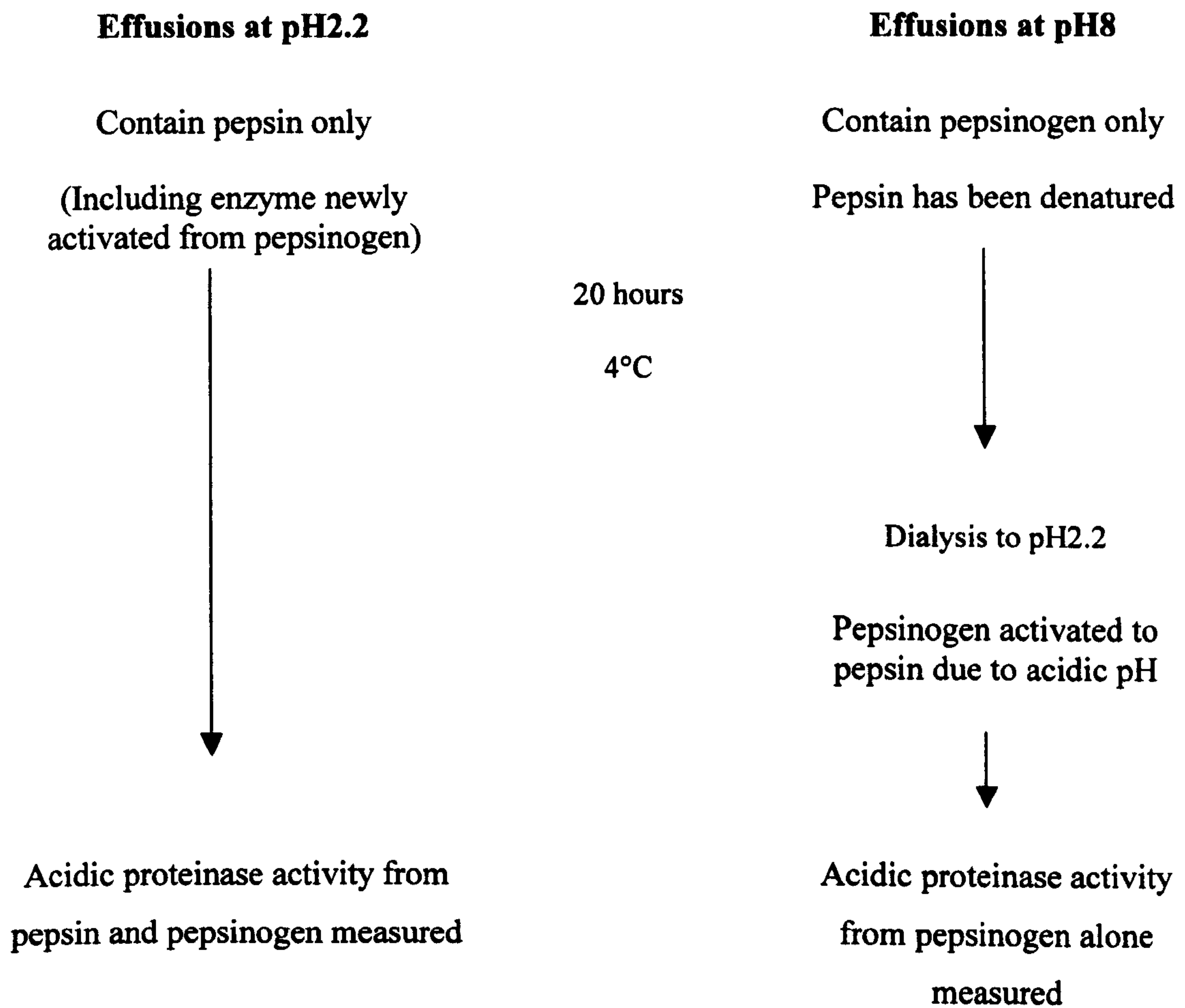
3.2.1 Middle ear effusion preparation

Middle ear effusions (n=65) collected from children undergoing myringotomy for OME (at Newcastle and Nottingham ENT) and stored at -20°C were thawed at 4°C before use. The samples were randomly chosen from storage and therefore varied in their type (from serous to mucoid) and also in their colour (from straw coloured to deep red).

The effusions were divided into two sets, one containing 35 effusions and the other containing 30 effusions and each effusion volume (μl) was recorded. To the first set (n=35) was added a known volume of 0.01M HCl (pH2.2) and to the second set (n=30) was added an equal volume of 0.067M phosphate buffer (pH8). Each effusion-acid or effusion-buffer (alkaline) mixture was gently homogenised and then centrifuged for 1 hour at 700g and 4°C to remove insoluble material. The supernatants were removed and stored at 4°C at their respective pHs for 20 hours. The pH8 supernatants were returned to pH2.2 by dialysis in 0.01M HCl before analysis of proteolytic activity (see section 2.3.1).

Pepsin has substantial proteolytic activity at pH2.2 and will be irreversibly inhibited at pH8 (Piper and Fenton, 1965). Pepsinogen is completely converted to pepsin at pH4.5 and below (Samloff, 1971). The effusions left at pH2.2 should therefore contain pepsin alone. Pepsinogen is stable at pH8-10 so treatment of an effusion supernatant at pH8 should irreversibly inhibit any pepsin present, and pepsinogen alone would survive. After the four hours dialysis at pH2.2 any pepsinogen present would be activated to pepsin. See diagram below.

These pH manipulations allow the calculation of pepsin content in effusions whilst accounting for the presence of serum pepsinogen.



3.2.2 Measurement of acidic proteinase activity in effusions

Proteolytic activity of the effusion supernatants was measured at pH2.2 using the N-terminal assay with succinyl albumin as substrate for proteolytic cleavage and porcine pepsin A as a standard (as described in section 2.4.1). Time zero controls were prepared to measure all free N-terminals on substrate and enzyme existing before hydrolysis.

In the assay, the amount of free N-terminals generated by the hydrolysis of peptide bonds from enzyme present in the effusion supernatant was quantified colorimetrically by the measurement of the optical density at 340nm of the final reaction volume. After subtracting the optical density values corresponding to the amount of N-terminals existing before hydrolysis and any reagent absorbance, acidic proteinase activity in 0.2ml of each effusion supernatant was calculated from the porcine pepsin standard curve.

The total amount of acidic proteinase activity per effusion and per ml effusion were calculated using the following formulae:

$$\begin{array}{l} \text{\textmu g acidic proteinase in 0.2ml} \times \frac{(\text{Original effusion (ml)} + \text{buffer / HCl added (ml)})}{0.2} = \text{\textmu g acidic proteinase activity per effusion} \\ \\ \frac{\text{\textmu g acidic proteinase activity per effusion}}{\text{effusion volume (\textmu l)}} \times 1000 = \text{\textmu g acidic proteinase activity per ml effusion} \end{array}$$

The pH8 to pH2.2 manipulation altered effusion volumes by a maximum of 2.5% therefore the original effusion volume was used to calculate pepsin and pepsinogen activity in all samples.

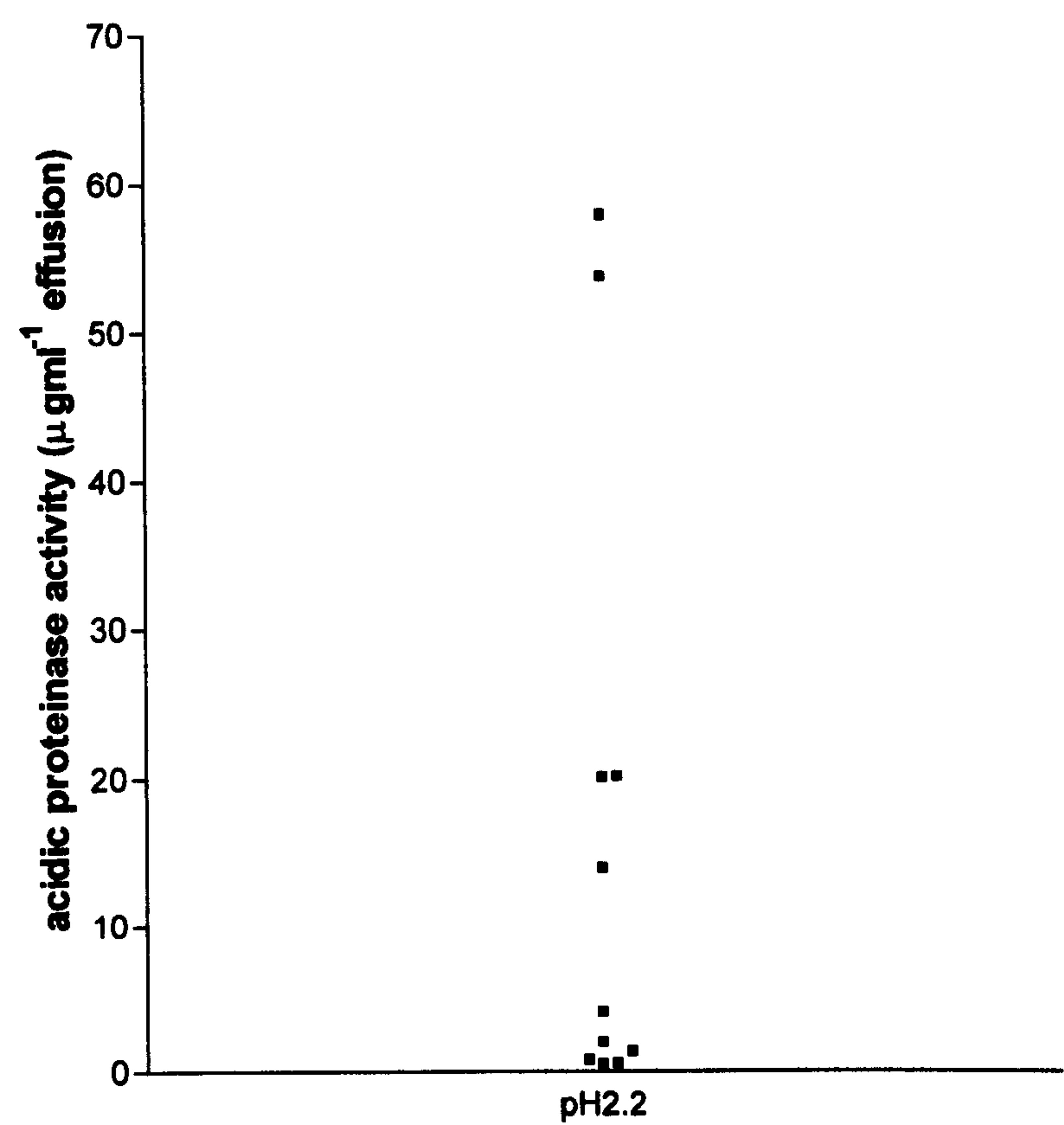
Thirty-five effusion supernatants were stored at pH2.2. Pepsin like activity (from pepsin and pepsinogen) was present in eleven (31%) of these. Activity ranged from 0.023–4.095µg acidic proteinase per effusion, which was equivalent to 0.551–57.93µg per ml effusion (Fig. 3.1).

Thirty effusion supernatants were exposed to pH8 buffer before being returned to pH2.2 for the assay. Pepsin-like activity (derived from pepsinogen) was found in eight (27%) of these. Activity ranged from 0.009–9.167µg acidic proteinase per effusion, equivalent to 3.996–147.4µg per ml effusion (Fig. 3.2).

It is unlikely that this activity is due to pepsinogen as serum pepsinogen levels are much lower (49–86ngml⁻¹) unless a massive concentration has occurred. The source cannot be gastric juice, as due to its low pH almost all the pepsinogen would have been already converted to pepsin.

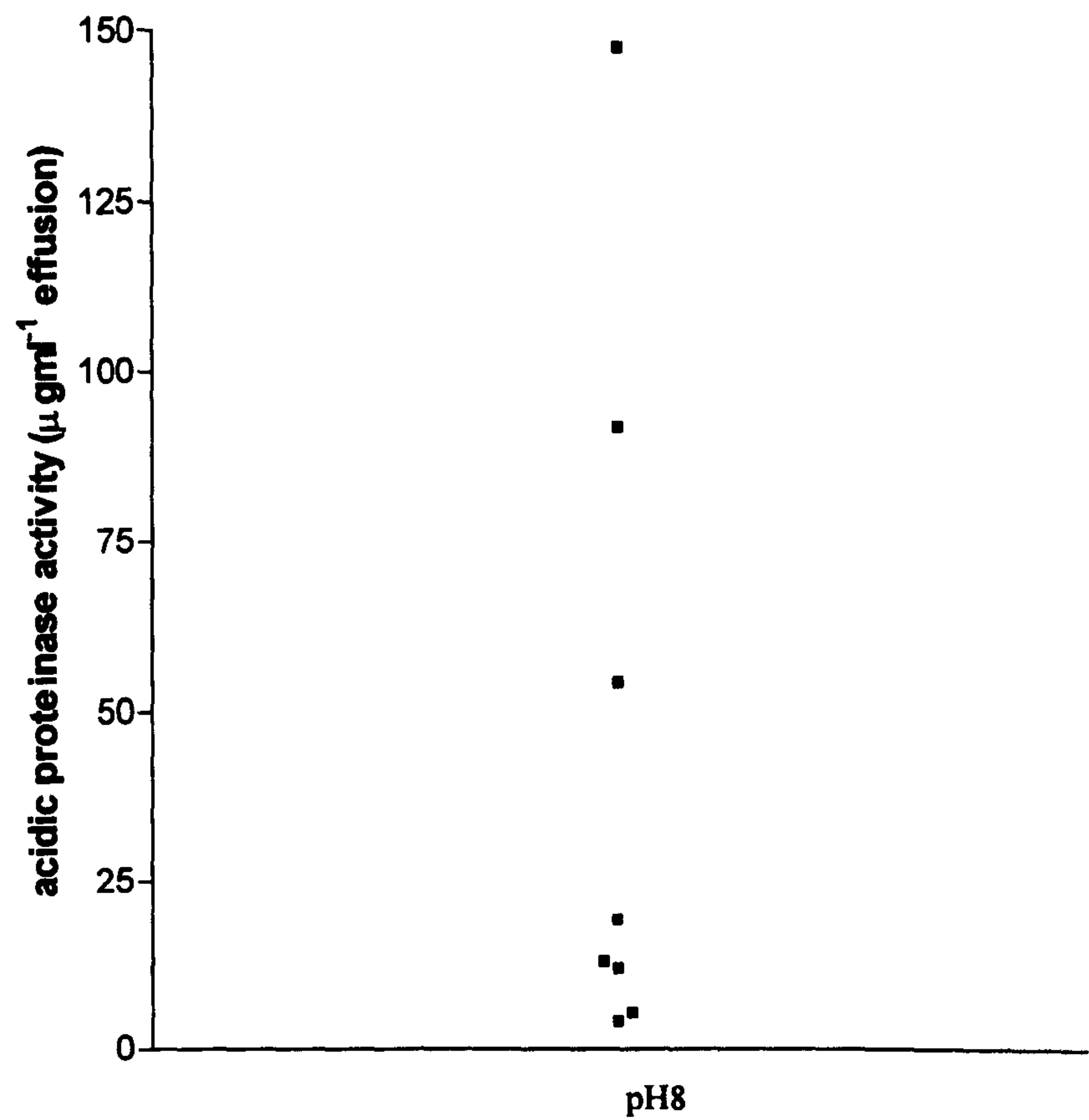
It is possible that exposure to pH8 is not sufficient to totally denature pepsin when in the presence of effusion or gastric juice components and that some pepsin activity remains when the proteinase activity of the effusion is subsequently measured at pH2.2. Bacteria have been cultured from up to 40% of middle ear effusions (Giebink, 1989; Bluestone *et al.*, 1992; Rayner *et al.*, 1998) therefore bacterial acidic proteinases could be present in the effusions, however an aspartate proteinase with this pH optimum has not yet been identified in bacteria.

Figure 3.1 Acidic proteinase activity (measured at pH2.2) per ml of effusion (homogenised in 0.01M HCl).



Effusion supernatants (■) stored at pH2.2 for 20 hours. Acidic proteinase activity was measured using the N-terminal assay.

Figure 3.2 Acidic proteinase activity (measured at pH2.2) per ml of effusion (homogenised in pH8 phosphate buffer).



Effusion supernatants (■) stored at pH8 for 20 hours and returned to pH2.2 by dialysis in 0.01M HCl for 4 hours. Acidic proteinase activity was measured using the N-terminal assay.

3.3 Acidic proteinase activity control assays

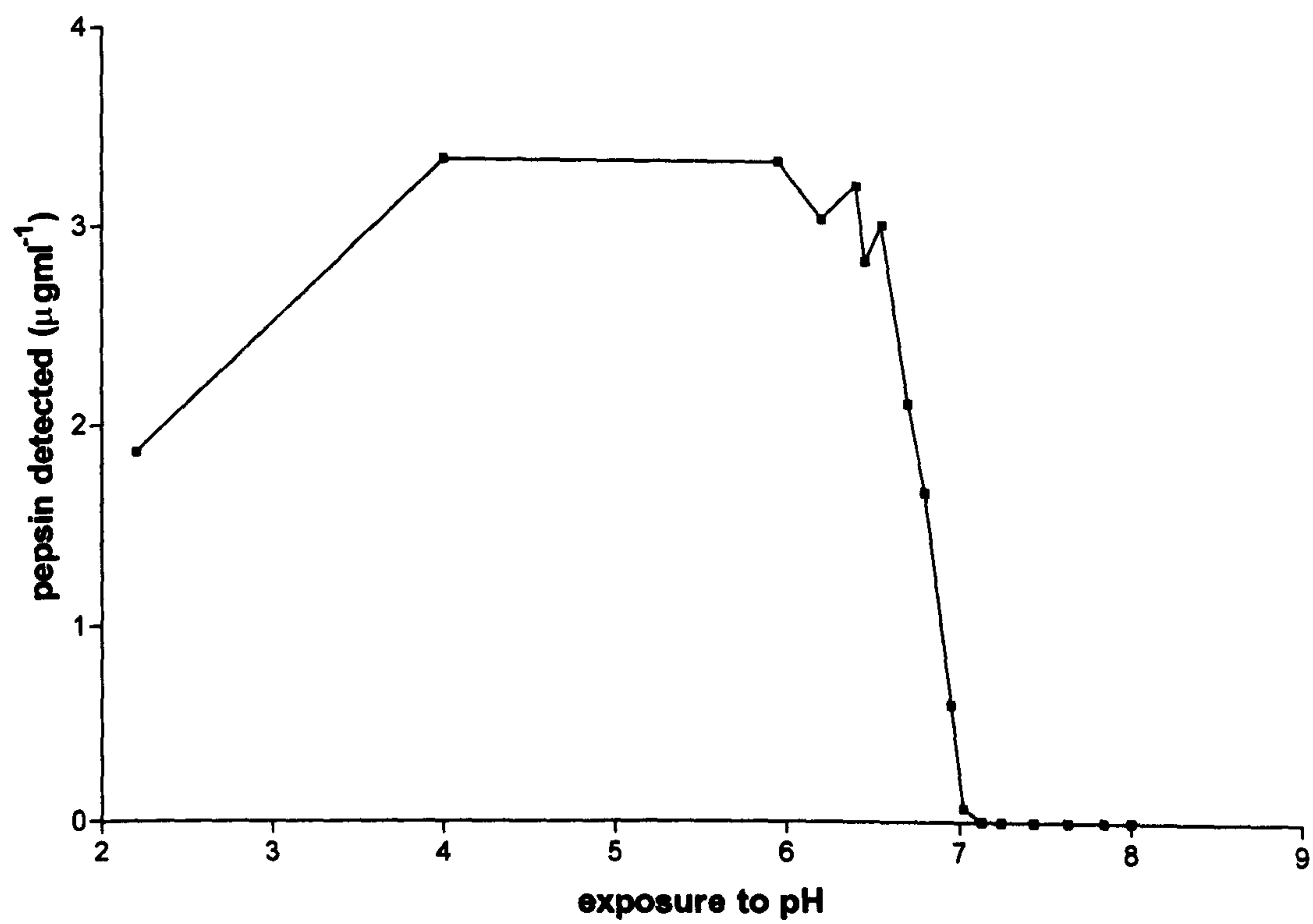
3.3.1 pH profile of porcine pepsin A

Pepsin is recognised as a proteolytic enzyme maximally active at a highly acidic pH and inactivated in neutral or alkaline solution. To investigate the exact pH at which porcine pepsin A is irreversibly denatured under the conditions used in the previous experiment, the enzyme was subjected to a range of pH and the remaining proteolytic activity was measured at pH2.2.

Sørensen's phosphate buffer (0.067M) was used in the range pH6.4 to pH8 with 0.1 or 0.2 pH unit increments. pH2.2 HCl was used as the control for maximum activity and dilute 0.0001M HCl (pH4) was used as an intermediate value. Porcine pepsin A was solubilised in acid or buffer at each pH to give solutions of $3.5\mu\text{gml}^{-1}$. The solutions were left for 20 hours at their respective pH and returned to pH2.2 by dialysis for 4 hours in 0.01M HCl. Proteolytic activity of the pepsin was measured using the N-terminal assay at pH2.2 with porcine pepsin A as standard.

It was observed that porcine pepsin A remained stable and retained all proteolytic activity when exposed to pH as high as pH6.6. After exposure to pH6.7, there was a 30% reduction in proteinase activity and by pH7.1 all activity was lost and porcine pepsin was irreversibly denatured (Fig. 3.3). A 42% reduction in activity was observed at pH2.2, the optimum pH for pepsin activity. This is likely to be due to auto-digestion of pepsin during the 20-hour pH exposure stage at 4°C before the assay. This reduction in temperature would not stop pepsin activity, it would just reduce the rate of activity (Piper and Fenton, 1965).

Figure 3.3 Levels of active pepsin (μgml^{-1}) detected in porcine pepsin solutions after exposure to a range of pH.



Porcine pepsin solutions (■) exposed to pH2.2-8 for 20 hours and returned to pH2.2 by dialysis in 0.01M HCl for 4 hours. Acidic proteinase activity was measured using the N-terminal assay.

3.3.2 pH profile of human gastric juice

To investigate whether pepsin present in gastric juice has the same pH profile as porcine pepsin A in solution.

Gastric juice samples were collected from patients under pentagastrin-stimulated conditions, diluted 1:10 with a pH4.1 buffer comprising 55mM sodium acetate and 55mM sodium chloride and stored at -20°C until used. For all experiments, gastric juice samples from different individuals were pooled.

Measurement of acidic proteinase activity (pH2.2)

The amount of pepsin activity at pH2.2 in the pooled gastric juice was measured in order that appropriate dilutions could be made for the pH profile assay. 1 in 5, 1 in 10 and 1 in 50 dilutions of gastric juice were made in 0.01M HCl. The amount of acidic proteinase activity present was measured using the N-terminal assay.

It was found that the concentration of active pepsin in the human gastric juice pool (1:10 dilution) was approximately 0.25mgml^{-1} and in the N-terminal assay system the 1 in 50 dilution of gastric juice was required in order to obtain results relevant to the porcine pepsin A standard curve.

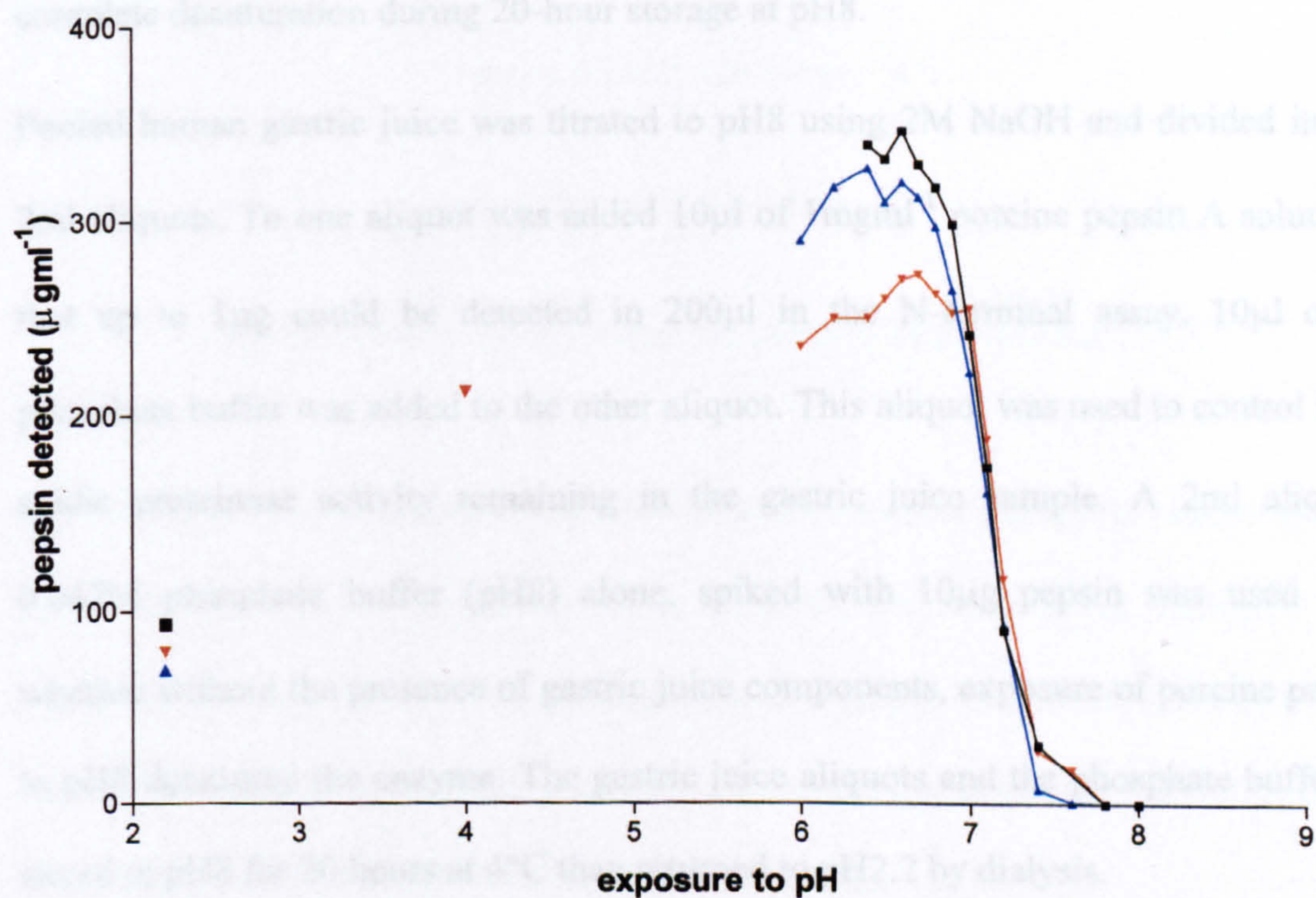
pH profile assay

Aliquots of the gastric juice pool were diluted in Sørensen's phosphate buffer (0.067M) in the range pH6.4 to pH8 with 0.1 or 0.2 increments and pH2.2 HCl was used as the control for maximum activity. The solutions were left for 20 hours at their respective pHs and returned to pH2.2 by dialysis for 4 hours in 0.01M HCl. Proteolytic activity

within the gastric juice was measured using the N-terminal assay at pH2.2 using porcine pepsin A as a standard. The pH profiles of three separate pools of human gastric juice were determined. In the third pool, dilute 0.0001M HCl (pH4) was used as an intermediate value.

It was observed that pepsin within the gastric juice remained stable and retained all its proteolytic activity when exposed to pH as high as pH7.0. After exposure to pH7.1 there was up to a 53% reduction in proteinase activity and by pH7.8 all activity was lost and human pepsin was irreversibly denatured (Fig. 3.4). A reduction in activity was observed at pH2.2 and at pH4 (only one sample measured at pH4). This is most likely due to auto-digestion. The reduction was more marked in these gastric juice samples than in the porcine pepsin solutions, this is most likely due to the higher concentration of enzyme present in the gastric juice samples.

Figure 3.4 Levels of active pepsin (μgml^{-1}) detected in three pooled gastric juice samples after exposure to a range of pH.



Gastric juice samples (pool 1 (■), pool 2 (▲) and pool 3 (▼)) were exposed to pH in the range pH2.2-8 for 20 hours and returned to pH2.2 by dialysis in 0.01M HCl for 4 hours. Acidic proteinase activity was measured using the N-terminal assay

3.3.3 Do components present in the assay protect pepsin from denaturation at pH8?

3.3.3.1 *Gastric juice components*

To investigate if the presence of gastric juice components protect porcine pepsin from complete denaturation during 20-hour storage at pH8.

Pooled human gastric juice was titrated to pH8 using 2M NaOH and divided into two 2ml aliquots. To one aliquot was added 10 μ l of 1mgml⁻¹ porcine pepsin A solution, so that up to 1 μ g could be detected in 200 μ l in the N-terminal assay. 10 μ l of pH8 phosphate buffer was added to the other aliquot. This aliquot was used to control for any acidic proteinase activity remaining in the gastric juice sample. A 2ml aliquot of 0.067M phosphate buffer (pH8) alone, spiked with 10 μ g pepsin was used to test whether without the presence of gastric juice components, exposure of porcine pepsin A to pH8 denatured the enzyme. The gastric juice aliquots and the phosphate buffer were stored at pH8 for 20 hours at 4°C then returned to pH2.2 by dialysis.

Acidic proteinase activity of the gastric juice (spiked and unspiked) and of porcine pepsin alone was measured using the N-terminal assay at pH2.2. See summary below.

<u>Gastric juice pH8</u>	<u>Gastric juice pH8</u>	<u>Phosphate buffer pH8</u>
10µl (10µg) porcine pepsin A added	10µl phosphate buffer (pH8) added	10µl (10µg) porcine pepsin A added

Stored at pH8, 20 hours, 4°C

Returned to pH2.2 by dialysis, 4°C

Acidic proteinase activity measured using N-terminal assay, pH2.2

The results of the N-terminal assay showed that even after storage of porcine pepsin A for 20 hours at a pH that is known to denature the enzyme, an additional 1.04% proteinase activity was detected in the gastric juice aliquot spiked with porcine pepsin A compared to gastric juice alone (Fig. 3.5). There was no proteinase activity detected in the phosphate buffer (pH8) spiked with porcine pepsin.

3.3.3.2 Middle ear effusion components

To investigate if the presence of middle ear effusion components protect porcine pepsin from complete denaturation during 20 hour storage at pH8.

Middle ear effusions (1.67mg) were pooled and diluted to 10ml final volume using 0.067M phosphate buffer, pH8. The mixture was hand homogenised and centrifuged for one hour (see method in section 2.3.1). The resulting supernatant was diluted as shown below.

Dilution	μg effusion material / 200 μl supernatant in assay
1 in 2	16.7
1 in 5	6.7
1 in 10	3.3

Each supernatant dilution was split into four equal aliquots of 2ml. Two aliquots from each dilution were used in this assay.

To one aliquot of each effusion dilution was added 10 μl of 1mgml⁻¹ porcine pepsin A solution, so that up to 1 μg could be detected in 200 μl in the N-terminal assay. 10 μl of pH8 phosphate buffer was added to the other aliquot. A 2ml aliquot of 0.067M phosphate buffer (pH8) alone, spiked with 10 μg pepsin was used to test whether without the presence of gastric juice components, exposure of porcine pepsin A to pH8 denatured the enzyme. All effusion dilutions and the phosphate buffer were stored at pH8 for 20 hours at 4°C then returned to pH2.2 by dialysis.

Acidic proteinase activity of the effusion dilutions (spiked and unspiked) and of porcine pepsin alone was measured using the N-terminal assay at pH2.2. See summary below.

<u>Effusion dilution pH8</u> (1 in 2, 1 in 5 & 1 in 10)	<u>Effusion dilution pH8</u> (1 in 2, 1 in 5 & 1 in 10)	<u>Phosphate buffer pH8</u>
10µl (10µg) porcine pepsin A added	10µl phosphate buffer (pH8) added	10µl (10µg) porcine pepsin A added

Stored at pH8, 20 hours, 4°C

Returned to pH2.2 by dialysis, 4°C

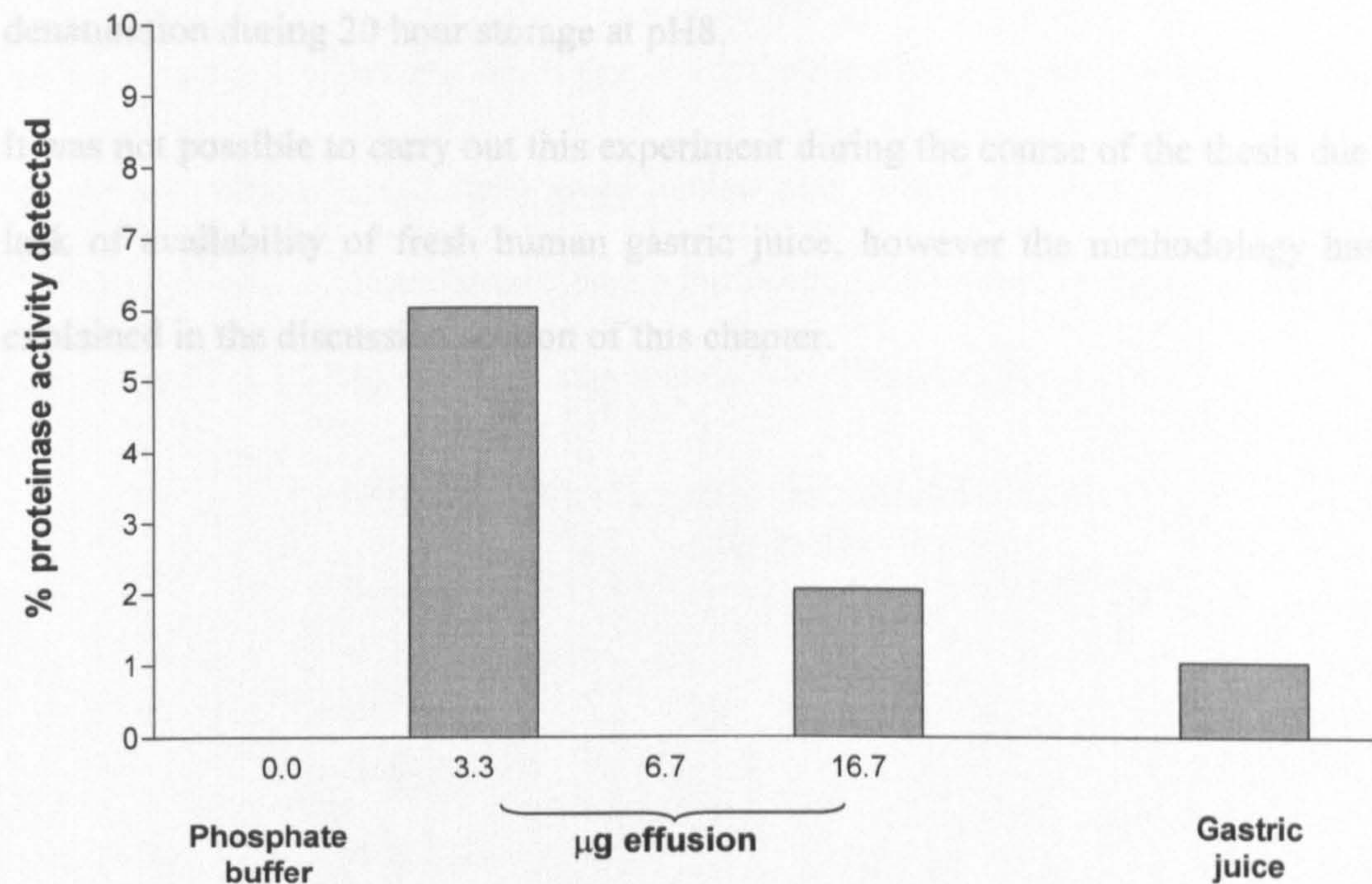
Acidic proteinase activity measured using N-terminal assay, pH2.2

After incubation of porcine pepsin with middle ear effusion supernatants containing varying amounts of effusion material, a small proportion, between 2.26 and 6.04% of proteinase activity remained (Fig. 3.5). There was no proteinase activity detected in the phosphate buffer (pH8) spiked with porcine pepsin.

Figure 3.5 Percentage of proteinase activity of porcine pepsin remaining after storage at pH8 with gastric juice or dilutions of middle ear effusion.

It was intended to investigate if the presence of both gastric juice components and middle ear effusion components protect human pepsins (in gastric juice) from denaturation during 20-hour storage at pH8.

It was not possible to carry out this experiment during the course of the thesis due to the unavailability of fresh human gastric juice, however the methodology has been described in the discussion section of this chapter.



The presence of gastric juice and middle ear effusion components prevent denaturation of porcine pepsin after storage at pH8 and dialysis to pH2.2. Proteinase activity was measured using the N-terminal assay.

3.3.3.3 *Gastric juice and middle ear effusion components*

It was intended to investigate if the presence of both gastric juice components and middle ear effusion components protect human pepsins (in gastric juice) from complete denaturation during 20 hour storage at pH8.

It was not possible to carry out this experiment during the course of the thesis due to the lack of availability of fresh human gastric juice, however the methodology has been explained in the discussion section of this chapter.

3.3.4 Does the amount of effusion material present in the N-terminal assay prevent 100% detection of pepsin?

To investigate if the presence of effusion contents in the N-terminal assay system was having a masking effect and inhibiting the detection of any pepsin present.

Middle ear effusions (1.17mg) were pooled and diluted to 11ml final volume using 0.01M HCl, pH2.2. The mixture was hand homogenised and centrifuged for one hour (see section 2.3.1). The resulting supernatant was diluted as shown below.

Dilution	µg effusion material / 200µl supernatant in assay
1 in 2	10.6
1 in 5	5.3
1 in 10	2.7
1 in 16	1.3

Middle ear effusions (1.67mg) were pooled and diluted to 10ml final volume using 0.067M phosphate buffer, pH8 (for preparation see section 3.3.3.2). The resulting supernatant was diluted as shown below.

Dilution	µg effusion material / 200µl supernatant in assay
1 in 2	16.7
1 in 5	6.7
1 in 10	3.3

The two 2ml aliquots from each dilution kept from the assay preparation described in section 3.3.3.2 were used in this assay. The aliquots were returned to pH2.2 by dialysis in 0.01M HCl for 4 hours at 4°C.

A 2ml aliquot of each supernatant dilution (from effusions homogenised both in pH2.2 and in pH8) was spiked with 10µl of 1mgml⁻¹ porcine pepsin A solution so that up to 1µg could be detected in 200µl in the N-terminal assay. To a second aliquot was added 10µl 0.01M HCl; this part was kept as a measurement of existing N-terminals.

Acidic proteinase activity of all sample dilutions, spiked and unspiked was measured using the N-terminal assay at pH2.2 with porcine pepsin A as standard. See summary below.

pH2.2 homogenised effusions		pH8 homogenised effusions	
2ml aliquot	2 ml aliquot	2ml aliquot	2ml aliquot
		Returned to pH2.2 by dialysis (0.01M HCl, 4 hours, 4°C)	
10µl (10µg) porcine pepsin A added	10µl 0.01M HCl (pH2.2) added	10µl (10µg) porcine pepsin A added	10µl 0.01M HCl (pH2.2) added

Stored at pH2.2, 20 hours, 4°C

Acidic proteinase activity measured using N-terminal assay, pH2.2

pH2.2 homogenised effusions

It was found that the unspiked pooled effusions did not contain any acidic proteinase activity and that all activity measured was therefore due to the spiked pepsin. There was an inverse correlation between the amount of effusion material present in the assay and the amount of pepsin that could be detected, demonstrating that effusion contents did reduce the amount of activity detected (Fig. 3.6). This correlation was statistically significant, $P=0.0037$, $r^2=0.958$. The weight of the average effusion used for measurement of acidic proteinase activity (section 3.2.1) was approximately 82µg (n=65), which after dilutions are made corresponded to 4.68µg per 200µl in the assay. This could mean that the effusion contents present are only allowing between 34 and 67% of acidic proteinase activity due to pepsin to be detected in the N-terminal assay system.

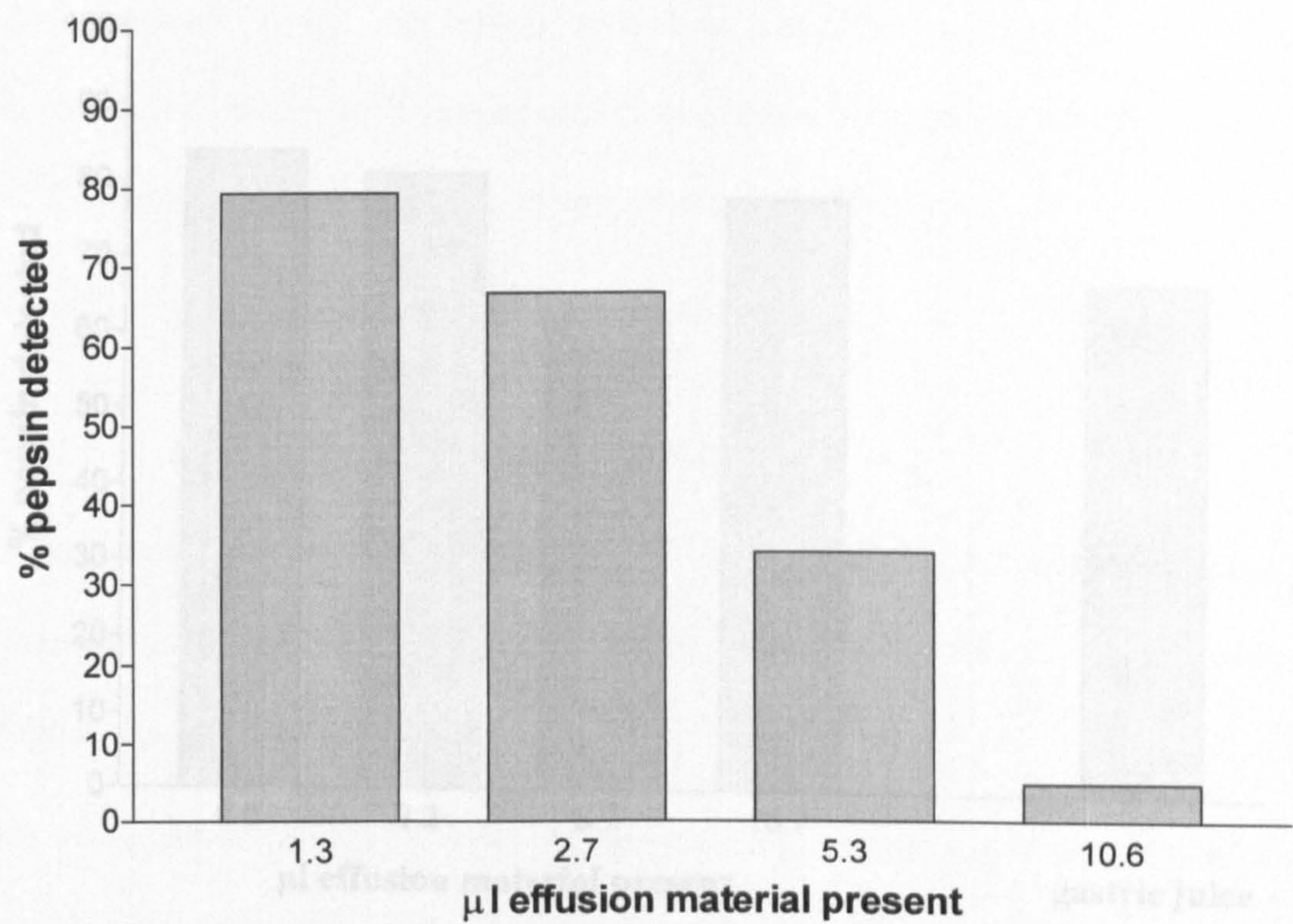
pH8 homogenised effusions

An inverse correlation was also observed as above between the amount of effusion material present and the percentage of spiked pepsin that could be detected. This effusion material was not as effective in preventing the pepsin being detected (Fig. 3.7). This is presumably due to solubilisation of the effusion at pH8 and then taking it to pH2.2. It is possible that pH8 treatment of the effusion reduces any interference properties and therefore could reduce any interference in the detection of the pepsin.

With the estimated effusion content in the assay (n=65) being approximately 4.68µg per 200µl, between 64 and 81% of the added porcine pepsin was detected. Gastric juice appeared to have a similar effect as the effusion material, with 67% of the added pepsin being detected (Fig. 3.7).

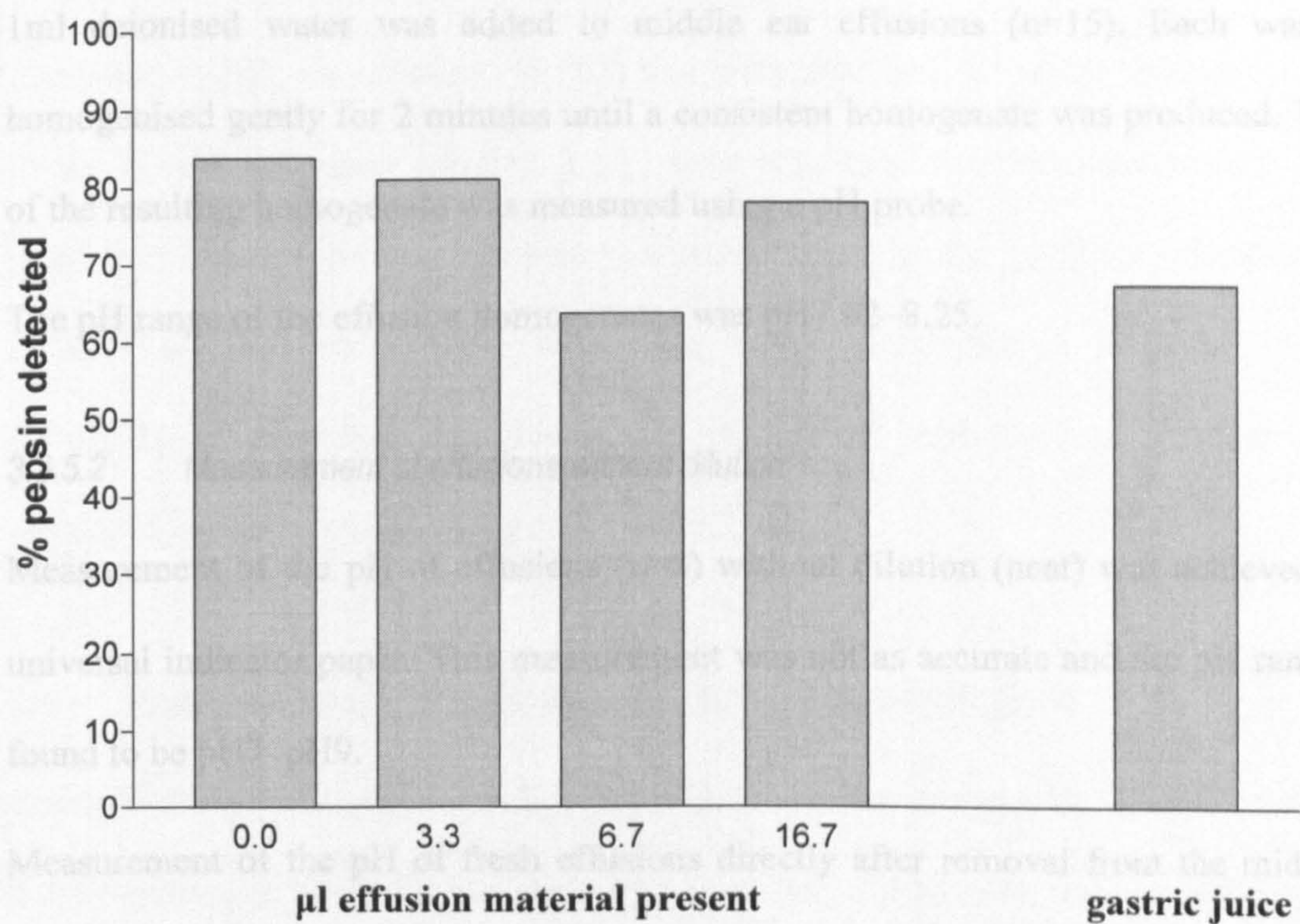
The masking effect of pepsin by effusion material was greater when the effusion was solubilised at pH2.2 rather than at pH8. The pH manipulation reduced the ability of the effusion components to interfere in the N-terminal assay.

Figure 3.6 Percentage of added pepsin detected in 200µl effusion supernatant containing a range of effusion material (pH2.2).



Effusions were homogenised in 0.01M HCl. Dilutions were made of the homogenate supernatant after centrifugation to obtain a range of effusion material. A known amount of porcine pepsin was added to each dilution and the percentage detected using the N-terminal assay was calculated.

Figure 3.7 Percentage of added pepsin detected in 200µl effusion supernatant containing a range of effusion material (pH8).



Effusions were homogenised in phosphate buffer, pH8. Dilutions were made of the homogenate supernatant after centrifugation to obtain a range of effusion material. Samples were returned to pH2.2 by dialysis and a known amount of porcine pepsin was added to each dilution and also to human gastric juice (pH2.2) and the percentage detected using the N-terminal assay was calculated.

3.3.5 pH of middle ear effusions

3.3.5.1 *Measurement in deionised water*

1ml deionised water was added to middle ear effusions (n=15). Each was hand homogenised gently for 2 minutes until a consistent homogenate was produced. The pH of the resulting homogenate was measured using a pH probe.

The pH range of the effusion homogenates was pH7.42–8.25.

3.3.5.2 *Measurement of effusions without dilution*

Measurement of the pH of effusions (n=8) without dilution (neat) was achieved using universal indicator paper. This measurement was not as accurate and the pH range was found to be pH7–pH9.

Measurement of the pH of fresh effusions directly after removal from the middle ear had the same pH range (Prof. J.P. Birchall, personal communication).

As shown by the pH profile of pepsin in gastric juice, some pepsin activity may be retained at these pHs, particularly between pH7-7.6.

3.4 Analysis of middle ear effusions for total pepsin/pepsinogen protein

3.4.1 Middle ear effusion preparation

The same middle ear effusions (n=65) that were analysed for acidic proteinase activity in the N-terminal assay (section 3.2.1) were also used for the measurement of total pepsin/pepsinogen protein content. Effusion supernatants (pH2.2) remaining after aliquots were taken for the N-terminal assay were diluted a further 1:2 and 1:5 in 0.01M phosphate buffered saline, pH7.4 and used for determination of total pepsin/pepsinogen protein using a slot blot ELISA.

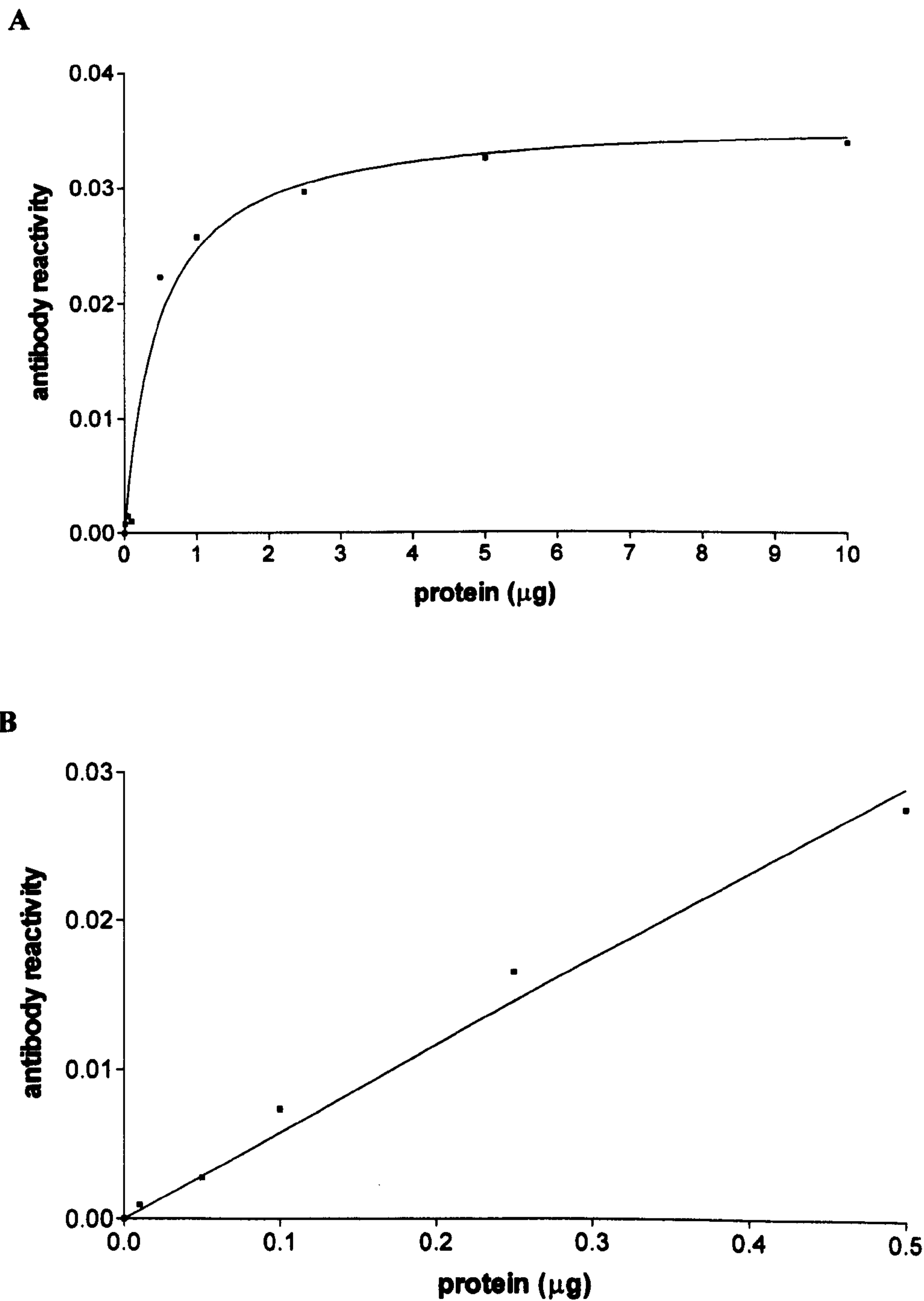
3.4.2 Measurement of total pepsin/pepsinogen protein in effusions

Pepsin/pepsinogen content of effusions was determined using a slot blot ELISA with a 0.2µm pore size nitrocellulose membrane (section 2.4.2) with porcine pepsin A as a standard (Fig. 3.8).

100µl of samples and standards were added to individual wells and allowed to absorb onto the membrane under vacuum. The membrane was probed with a monospecific antiserum raised to pepsin (porcine stomach), (Biodesign International, USA) at a dilution of 1:2000. This antibody reacts strongly with human pepsin (Ms. M. Panetti, personal communication) and recognises the same epitope in pepsinogen as in pepsin so cannot differentiate between precursor and active enzyme. Antibody binding was measured using the appropriate secondary antibody (anti-goat) conjugated to peroxidase with H₂O₂ as substrate. 3,3'-diaminobenzidine tetrahydrochloride (DAB) was used for colour development. Staining of the slots was quantitated at 595nm.

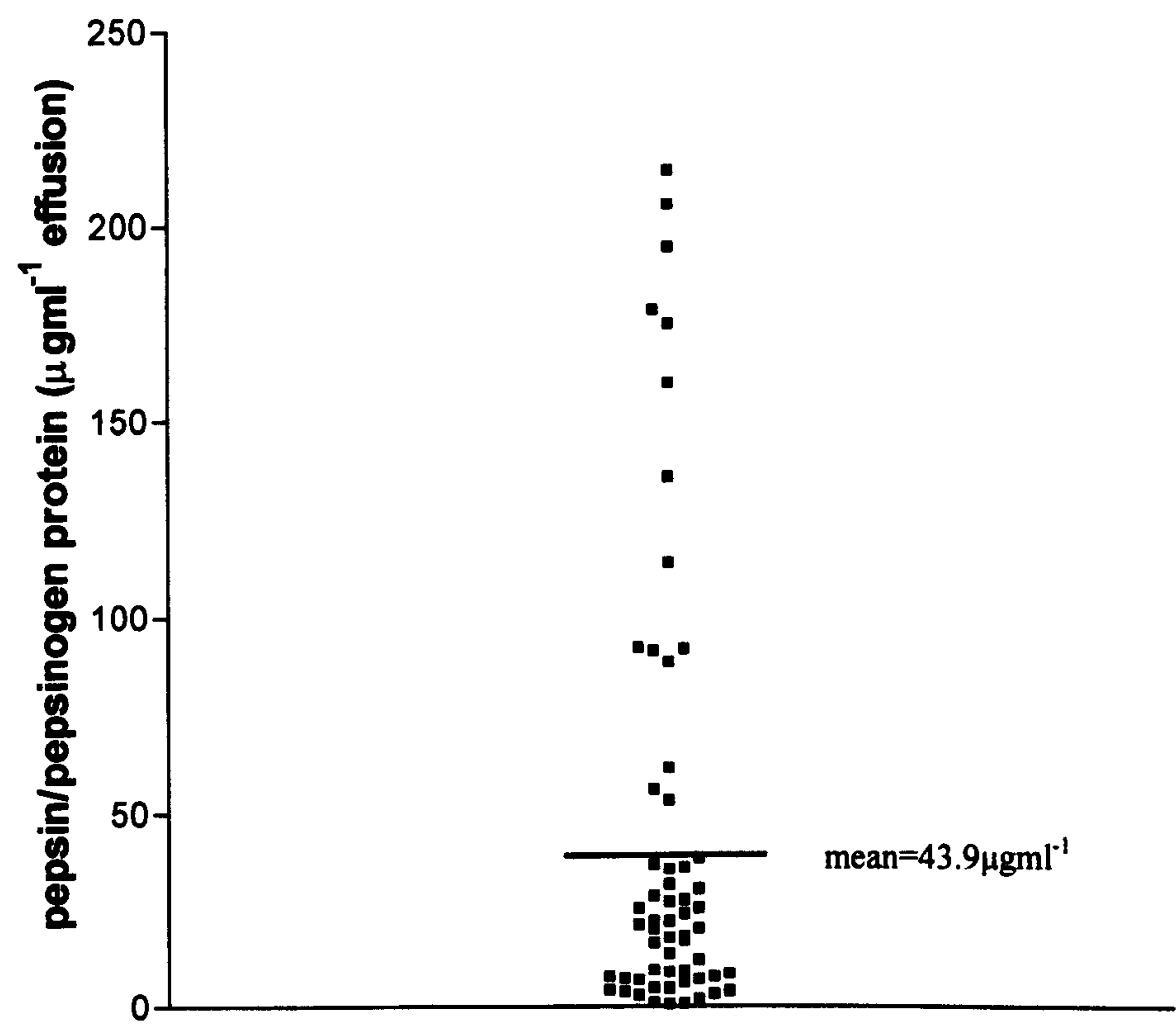
Fifty-nine of sixty-five effusions (91%) gave a positive result with the anti-pepsin antibody. Total pepsin/pepsinogen protein present in the 100µl of each effusion on the membrane was calculated using the porcine pepsin A standard curve. Pepsin/pepsinogen protein per ml of effusion was calculated taking into account all the dilution steps made and was in the range 0.8–213.9µg/ml effusion (mean=43.9; SD=57.2) for the positive effusions (n=59) (Fig. 3.9 and 3.10). 77% of effusions contained less than 50µg pepsin/pepsinogen per ml effusion, demonstrating that only a small number of effusions (n=15) had a very high pepsin/pepsinogen content. Normal serum reference levels for pepsinogen are 49–86ngml⁻¹, these levels are much lower than those found in middle ear effusions.

Figure 3.8 Standard curves of porcine pepsin A.



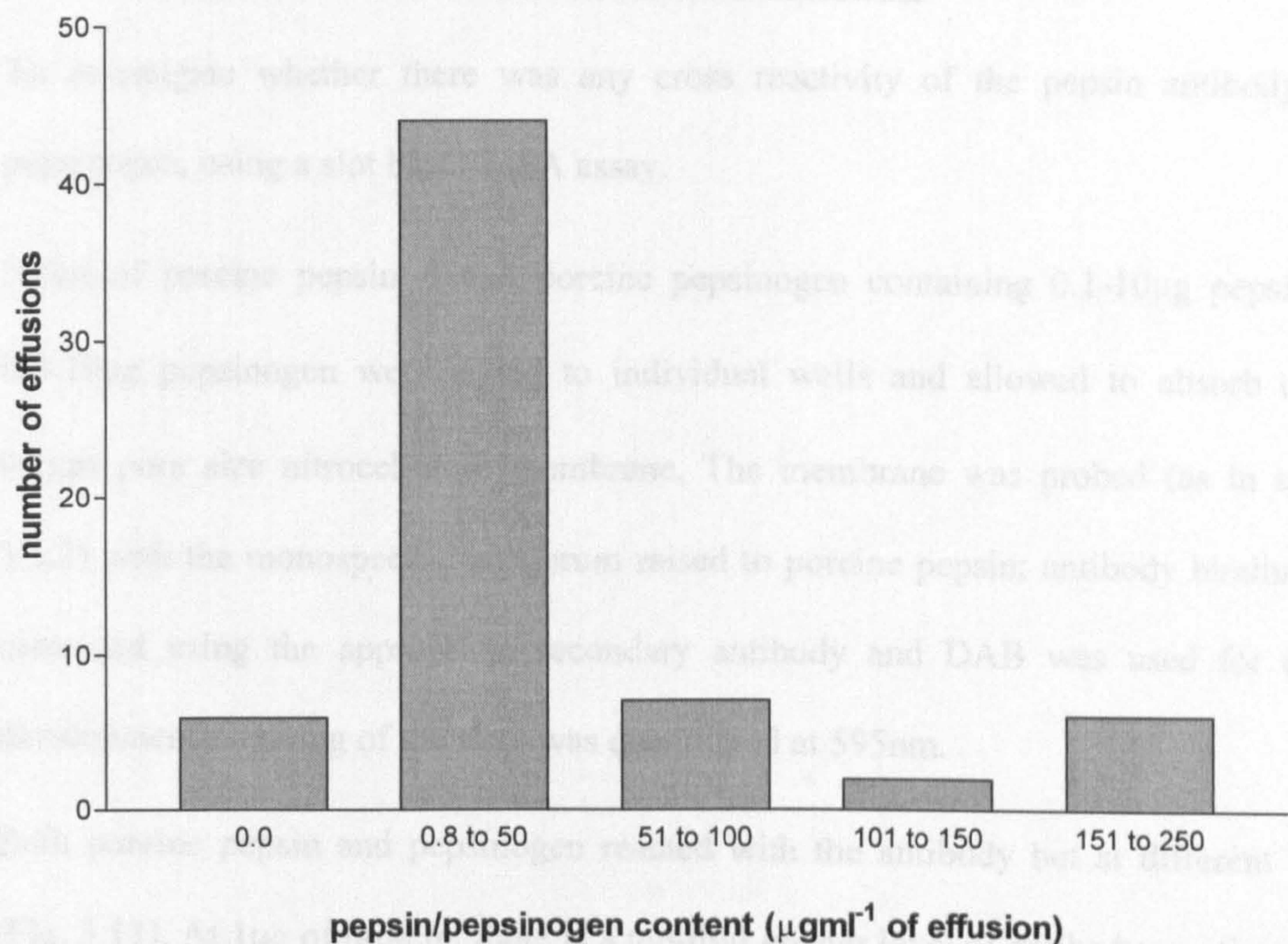
The porcine pepsin standard curve in the range 0.01-10µg protein had a distinct plateau occurring after 0.5µg per well (graph A). The range 0.01-0.5µg protein (graph B) was used to calculate pepsin/pepsinogen protein, as this graph was linear.

Figure 3.9 **Total pepsin/pepsinogen content in μg per ml of effusion.**



Total pepsin/pepsinogen content of effusions (■) was determined using an ELISA with porcine pepsin A as standard. 91% of effusions contained a detectable level of pepsin/pepsinogen protein in the range 0.8-213.9 μgml^{-1} effusion.

Figure 3.10 Total pepsin/pepsinogen content per ml of each effusion expressed by amount of protein present.



Total pepsin/pepsinogen content of effusions, demonstrating the distribution of the range of protein present.

3.5 Total pepsin/pepsinogen content control assays

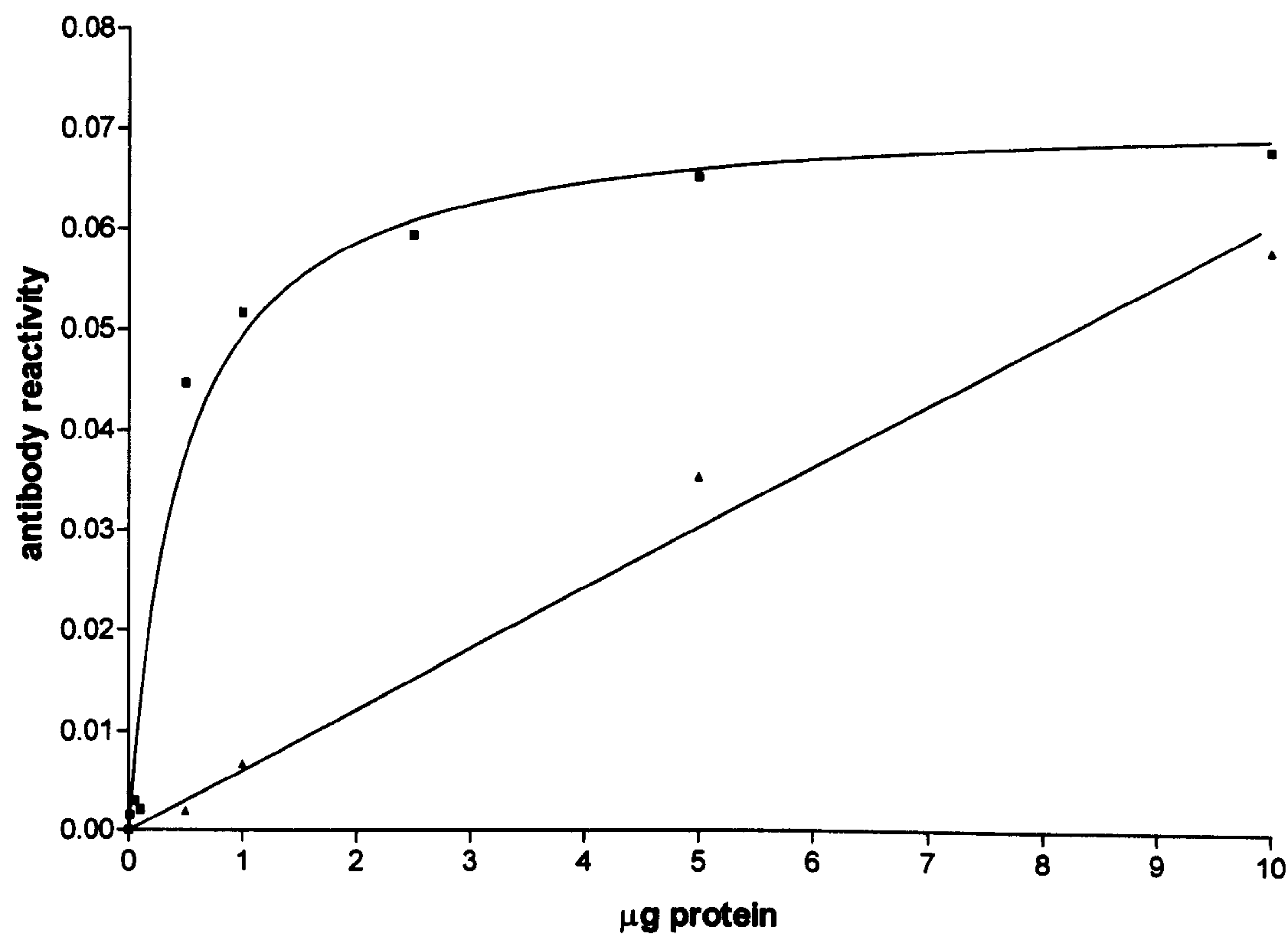
3.5.1 Cross reactivity of pepsinogen with pepsin antibody

To investigate whether there was any cross reactivity of the pepsin antibody with pepsinogen, using a slot blot ELISA assay.

100µl of porcine pepsin A and porcine pepsinogen containing 0.1-10µg pepsin and 0.5-10µg pepsinogen were added to individual wells and allowed to absorb onto a 0.2µm pore size nitrocellulose membrane. The membrane was probed (as in section 3.3.2) with the monospecific antiserum raised to porcine pepsin; antibody binding was measured using the appropriate secondary antibody and DAB was used for colour development. Staining of the slots was quantitated at 595nm.

Both porcine pepsin and pepsinogen reacted with the antibody but at different levels (Fig. 3.11). At 1µg of protein, there is a ten-fold greater level of antibody reactivity with porcine pepsin than porcine pepsinogen. The antibody could not differentiate between pepsin and pepsinogen and one must assume that both proteins are being measured simultaneously and that results represent the sum of the two proteins.

Figure 3.11 Reactivity of porcine pepsin and pepsinogen with the pepsin antibody



Standard curves of porcine pepsin A (0.1-10μg, ■) and porcine pepsinogen (0.5-10μg, ▲). The pepsin standard curve is saturated above 3μg protein yet the pepsinogen standard curve is still linear up to 10μg. This assay appears to be relatively insensitive to pepsinogen therefore one can assume that the positive protein in the effusions is derived from pepsin not pepsinogen.

3.5.2 Measurement of serum protein levels in effusions

As middle ear effusions are in part a transudate of plasma, two of the major serum proteins, fibrinogen and albumin, were measured in effusions as well as the pepsin/pepsinogen concentration using a slot blot ELISA assay. A comparison was made with normal serum levels to examine whether serum factors were being concentrated in the effusions. If the effusions were a ten-fold concentration of plasma, then the pepsinogen content could be in the range $0.5\text{--}0.9\ \mu\text{gml}^{-1}$, which is similar to the pepsinogen levels already measured in a small number of the effusions.

3.5.2.1 *Fibrinogen levels*

Known volumes of effusion supernatant ($n=8$) in PBS were blotted onto nitrocellulose sheets ($0.45\mu\text{m}$ pore size for fibrinogen, $0.2\mu\text{m}$ pore size for pepsin/pepsinogen) along with the relevant standards (fibrinogen (fraction I, human plasma) or pepsin A (porcine stomach)) and probed with either a polyclonal antiserum raised to human fibrinogen or the monospecific antibody raised to pepsin (porcine stomach), both at a 1:2000 dilution.

Antibody binding was measured using the appropriate secondary antibody (anti-rabbit or anti-goat IgG) conjugated to peroxidase with H_2O_2 as substrate. DAB was used for colour development as above.

All effusions contained fibrinogen in the range $0.30\text{--}2.30\text{mgml}^{-1}$ (mean=1.08; SD=0.69 ($n=8$)) (Fig. 3.12). The mean concentration of fibrinogen in the effusions was 1.077mgml^{-1} of effusion. Serum reference levels for fibrinogen are $2.2\text{--}4.6\text{mgml}^{-1}$. The pepsin/pepsinogen content of seven of the effusions ranged from $3.29\text{--}27.97\mu\text{gml}^{-1}$ (mean=18.32; SD=10.15 ($n=7$)). There was not enough sample remaining to measure

pepsin/pepsinogen content in the eighth effusion. These values are consistent with levels of pepsin/pepsinogen previously measured in effusions.

3.5.2.2 *Albumin levels*

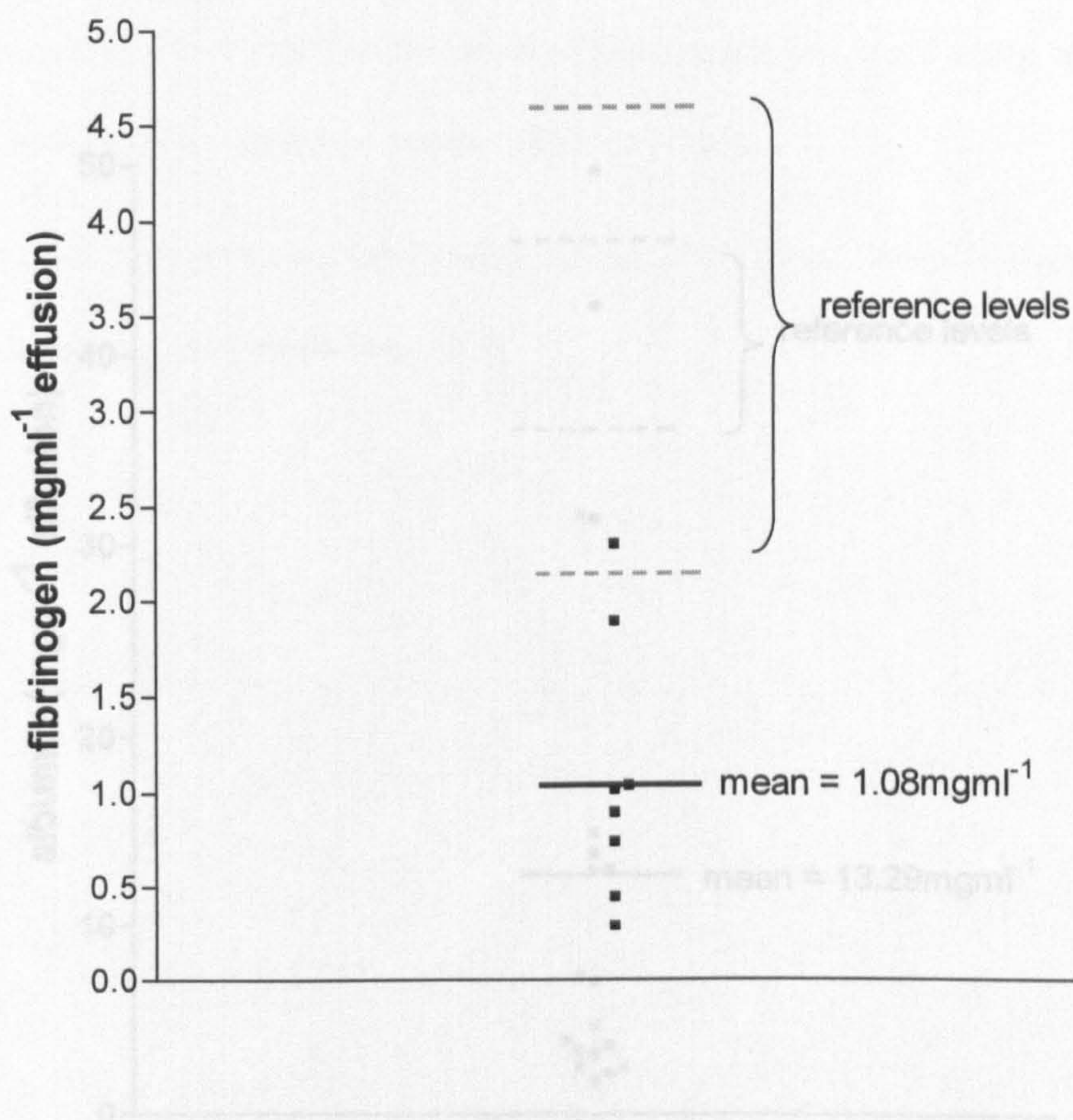
Known volumes of effusion supernatant (n=19) in PBS were blotted onto nitrocellulose sheets (0.45µm pore size for albumin, 0.2µm pore size for pepsin/pepsinogen) along with the relevant standards (albumin (fraction V, human plasma) or pepsin A (porcine stomach) and probed with either a monoclonal antiserum raised to human albumin used at a 1:5000 dilution or the monospecific antibody raised to pepsin (porcine stomach) at a 1:2000 dilution.

Antibody binding was measured using the appropriate secondary antibody (anti-mouse or anti-goat IgG) conjugated to peroxidase with H₂O₂ as substrate. DAB was used for colour development as above.

All effusions contained albumin in the range 1.78–49.7mgml⁻¹ (mean=13.29; SD=14.66 (n=19)) (Fig. 3.13). The mean concentration of albumin in the effusions was 13.29mgml⁻¹ of effusion. Serum reference levels for albumin are 35-45mgml⁻¹.

The pepsin/pepsinogen content of four of the above effusions ranged from 5.89–25.35µgml⁻¹ (mean=16.05; SD=7.96 (n=4)). These values are consistent with levels of pepsin/pepsinogen previously measured in effusions.

Figure 3.12 Fibrinogen content (mg) per ml effusion.



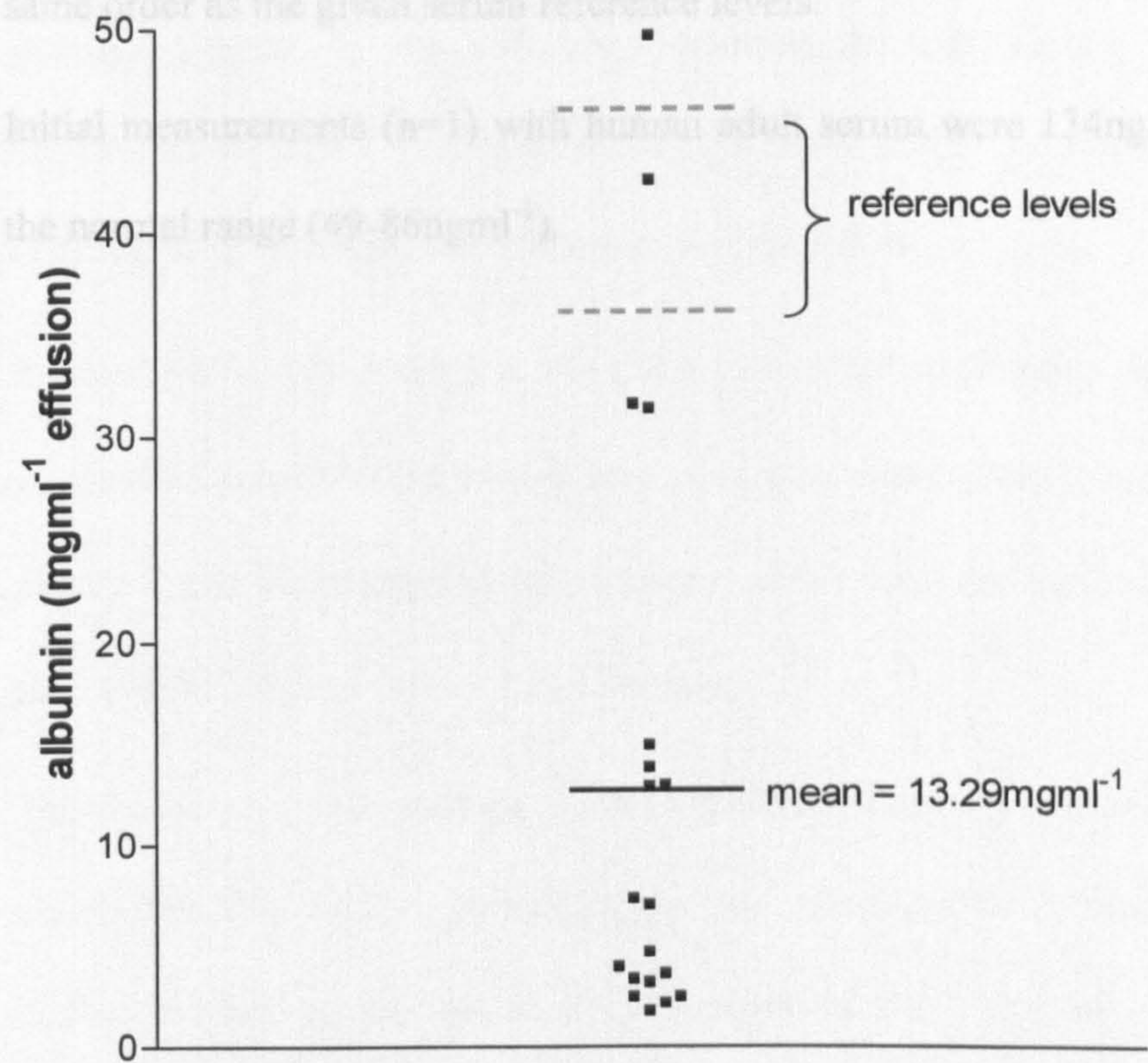
Fibrinogen content of effusions (■) was determined using an ELISA with human fibrinogen as standard. All effusions contained a detectable level of fibrinogen in the range 0.3–2.3mgml⁻¹ effusion. Serum reference levels are 2.2–4.6mgml⁻¹ (---).

Figure 3.13 Albumin content (mg) per ml effusion.

3.5.3 Measurement of serum pepsinogen levels using pepsin antiserum

This was used to measure the reactivity of the pepsin antiserum with pepsinogen from serum and to verify that the level of pepsinogen measured using the antibody was of the same order as the given serum reference levels.

Initial measurements ($n=5$) with human serum were 13.4mgml^{-1} and were close to the normal range ($4.7\text{--}36\text{mgml}^{-1}$).



Albumin content of effusions (■) was determined using an ELISA with human albumin as standard. All effusions contained a detectable level of albumin in the range $1.78\text{--}49.7\text{mgml}^{-1}$ effusion. Serum reference levels $35\text{--}45\text{mgml}^{-1}$ (— — —).

3.5.3 Measurement of serum pepsinogen levels using pepsin antiserum

This was used to measure the reactivity of the pepsin antiserum with pepsinogen from serum and to verify that the level of pepsinogen measured using the antibody was of the same order as the given serum reference levels.

Initial measurements (n=1) with human adult serum were 134ngml^{-1} and were close to the normal range ($49\text{-}86\text{ngml}^{-1}$).

3.5.4 Does the pepsin antibody react with other serum proteins?

3.5.4.1 *Measurement of pepsin antibody reactivity with serum proteins*

It was possible that the pepsin monospecific antiserum was also reacting with other proteins present in the effusion, resulting in high values for pepsin/pepsinogen (compared to serum levels) even though other serum protein levels were of similar concentration in serum and effusions (section 3.5.2).

Standard curves (1-200 μ g) of the main human serum proteins, albumin, fibrinogen and γ -globulins were blotted onto 0.2 μ m pore size nitrocellulose sheets along with porcine pepsin A and pepsinogen standard curves (0.0-1.0 μ g) and probed with the monospecific pepsin antibody in a slot blot ELISA assay.

There was a small amount of non-specific reactivity observed with albumin and γ -globulins that was not concentration dependent. Antibody binding of 3.5% and 0.9% of that for 1 μ g of porcine pepsin was observed for 200 μ g of γ -globulins and albumin respectively. However, there was concentration dependent cross reactivity between the pepsin antibody and fibrinogen (Fig. 3.14a). Antibody binding of 20% of that for 1 μ g of porcine pepsin was observed for 200 μ g of fibrinogen (Fig 3.14a, Fig 3.14b).

3.5.4.2 *Determination of purity of serum protein preparations*

There was potential that the antibody reactivity observed with fibrinogen and γ -globulins was due to impurities present in the preparations. To test this possibility, samples of each protein were separated using SDS PAGE and stained with coomassie blue, a protein stain, to see if there was any protein impurity present in the fibrinogen or γ -globulin preparation that had a molecular weight similar to pepsin, (M_r =34500).

Albumin cross-reactivity had been very small and therefore was not investigated any further.

1mgml⁻¹ solutions of fibrinogen, γ -globulin, porcine pepsin A and molecular weight markers (range 14300 to 65000) were made in non-reducing buffer (0.0625M Tris buffer, pH6.8, containing 2% (w/v) SDS, 10% (v/v) glycerol and 0.001% bromophenol blue (see section 2.6.2 for complete procedure)). Solutions were heated at 100°C for 2 minutes and 1 μ l applied to polyacrylamide gels (Phastgel™ Gradient 4-15%). Gels were run at constant current until the tracking dye reached the end of the running gel. Gels were stained for protein using 0.05% (w/v) coomassie blue, 25% (v/v) propan-2-ol and 10% (v/v) acetic acid. After destaining in 25% (v/v) propan-2-ol and 10% (v/v) acetic acid followed by 10% (v/v) propan-2-ol and 10% (v/v) acetic acid, gels were scanned for protein at 585nm using a scanning densitometer at 585nm.

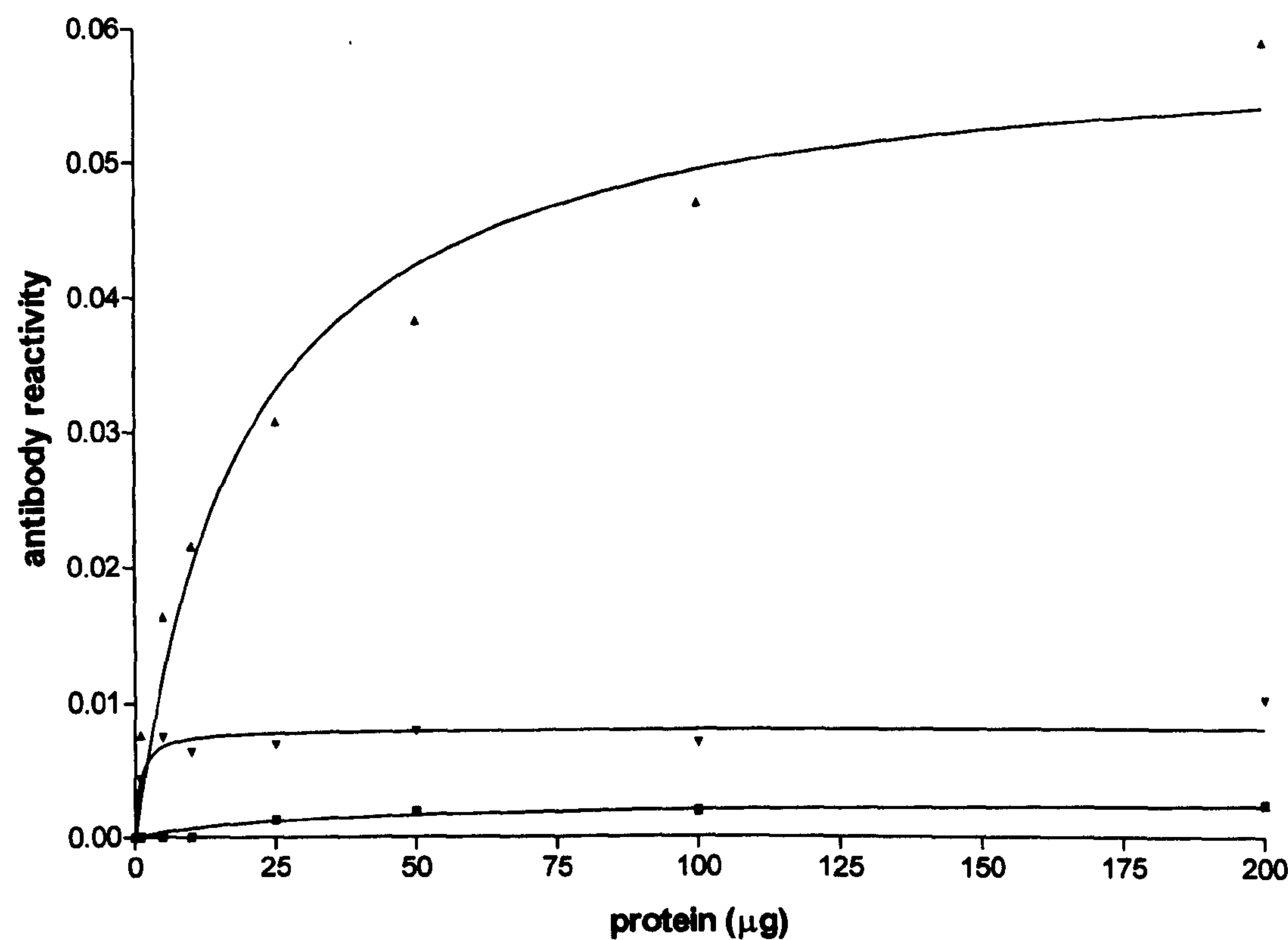
There were no detectable impurities present in either the γ -globulin or the fibrinogen preparations (Fig. 3.15) and nothing was observed at the molecular weight for pepsin therefore the antibody reactivity observed in the slot blot ELISA cannot be due to contamination of the proteins with pepsin/pepsinogen.

3.5.4.3 *Is the pepsin antibody detecting fibrinogen?*

In a previous experiment (described in section 3.5.2), both the fibrinogen and pepsin/pepsinogen content of effusions (n=7) was measured. The Pearson correlation between the two parameters was calculated, P value=0.3929 and r^2 =0.1487 (Fig. 3.16). There was no significant correlation between the two protein levels in these effusions, therefore the pepsin antibody could not be recognising fibrinogen alone. If the pepsin

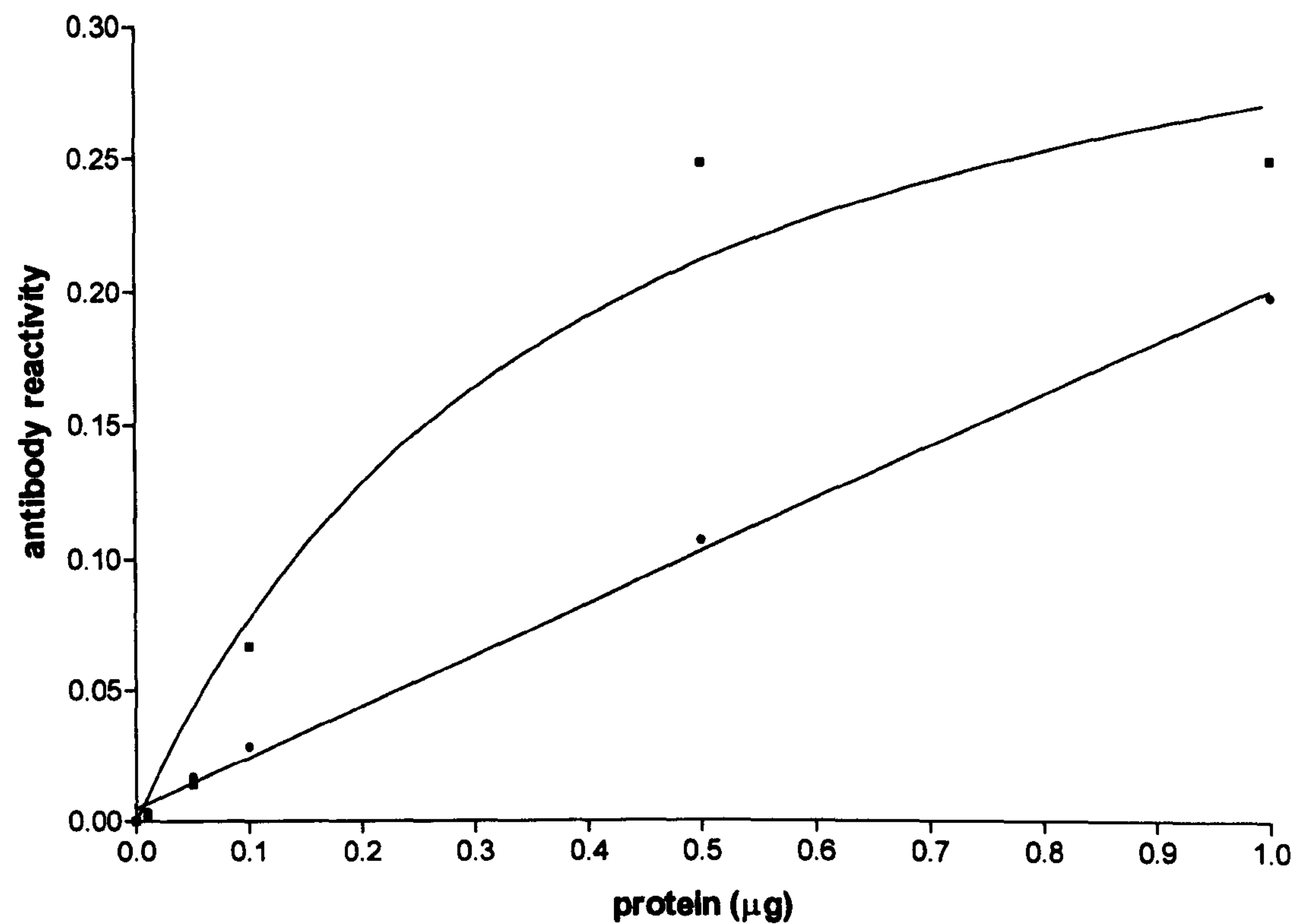
antibody was mainly reacting with fibrinogen, the correlation would be expected to be statistically significant.

Figure 3.14a Reactivity of serum proteins with pepsin antibody.



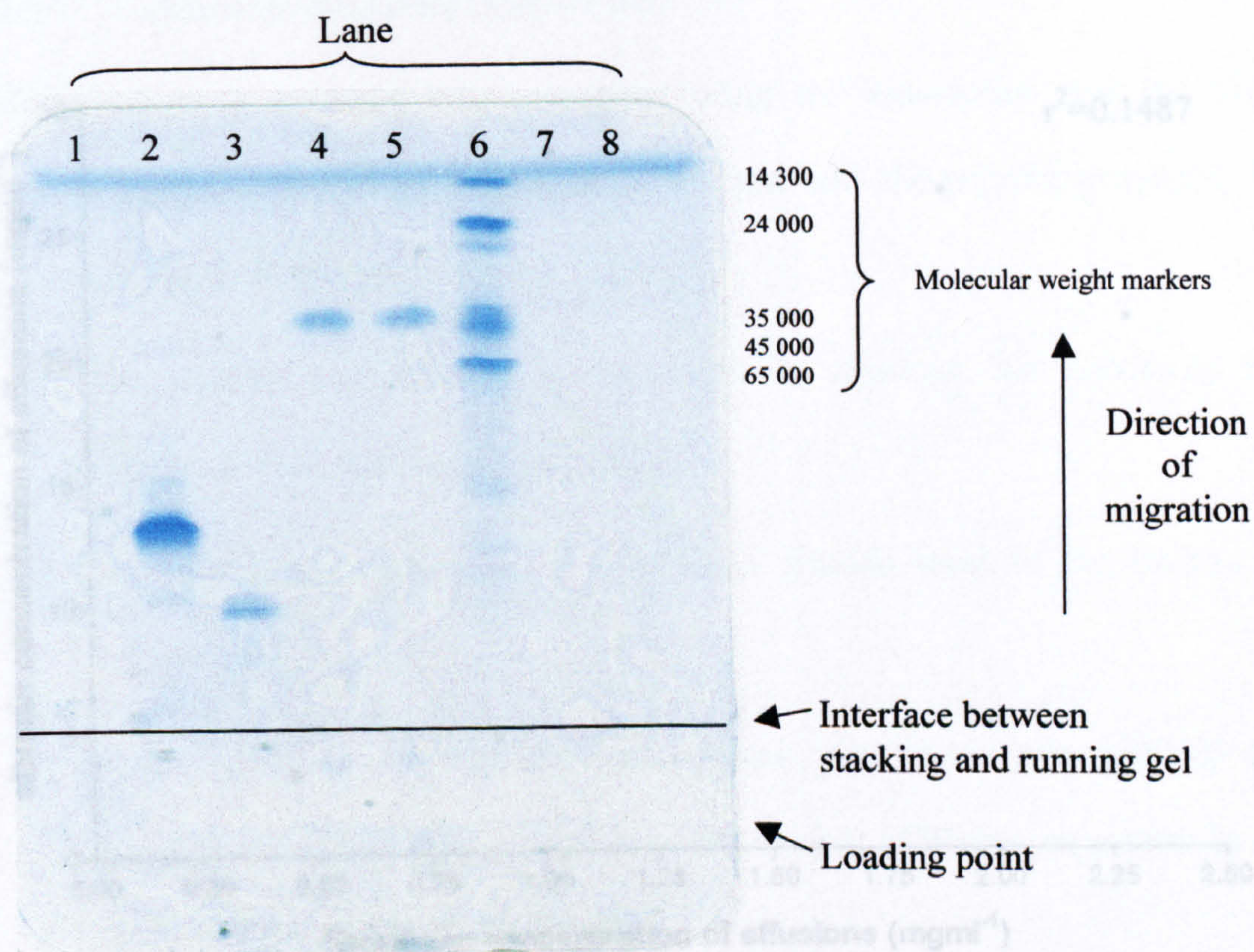
Concentration dependent reactivity was observed with fibrinogen (▲), non-specific and concentration independent reactivity was seen with γ -globulins (▼) and albumin (■) with the pepsin antibody in an ELISA assay.

Figure 3.14b Reactivity of porcine pepsin A and pepsinogen with pepsin antibody.



Concentration dependent reactivity was observed with both pepsin (■) and pepsinogen (●) with the pepsin antibody in an ELISA assay. These standard curves were used as positive controls and also to measure the difference in antibody binding between the pepsin and pepsinogen and the serum proteins.

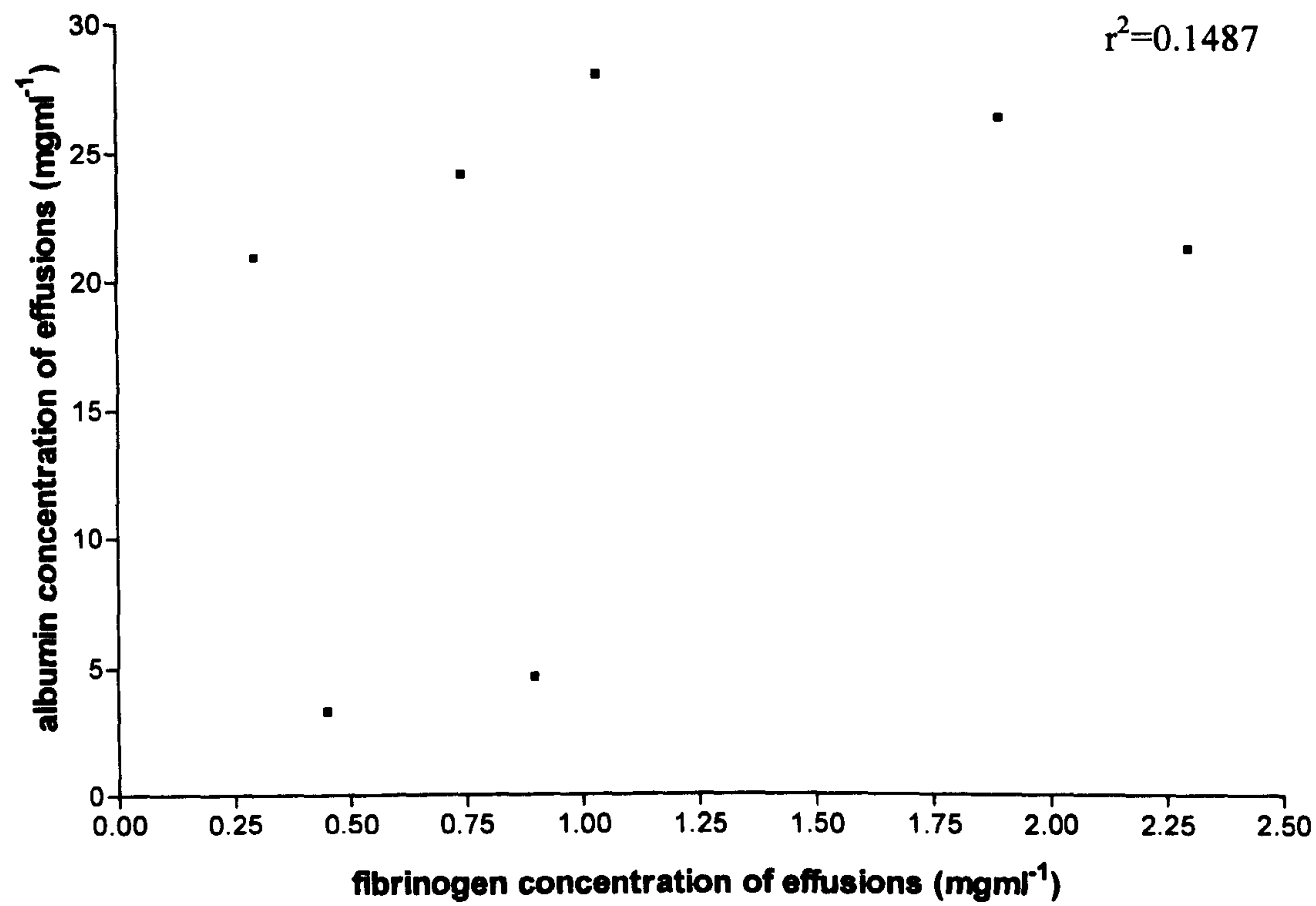
Figure 3.15 Separation of serum proteins, pepsin and molecular weight markers by SDS PAGE.



Lane	Loading
2	γ -globulins (1 μ g)
3	Fibrinogen (1 μ g)
4	Pepsin (1 μ g)
5	Pepsin (1 μ g)
6	Molecular weight markers

No protein impurities were observed in either the γ -globulin or fibrinogen preparations at the molecular weight for pepsin.

Figure 3.16 Comparison of pepsin/pepsinogen content and fibrinogen content of effusions



There was no correlation between the two parameters (P value=0.3929, Pearson correlation, n=7) therefore the pepsin antibody does not recognise fibrinogen alone.

3.5.5 Calculation of degree of cross reactivity between pepsin antibody and fibrinogen

3.5.5.1 *Calculated (theoretical) cross reactivity*

Theoretical cross reactivity was calculated using the assumption that the pepsin antibody would react with all fibrinogen present and with the pepsin/pepsinogen. This was estimating a 'worst case scenario'.

1. The average concentration of fibrinogen in effusions had previously been calculated to be 1.08mgml^{-1} (Fig. 3.12).
2. The fibrinogen content in each effusion dilution used in the ELISA was calculated.
3. From the fibrinogen standard curve probed with the pepsin antibody (Fig. 3.14a), antibody-binding equivalent to the amount of fibrinogen present in each effusion dilution was calculated.
4. The pepsin antibody binding equivalent to the presence of fibrinogen was subtracted from the total pepsin antibody binding per effusion, leaving the antibody binding relating to pepsin/pepsinogen presence in each effusion dilution.
5. Using the corresponding standard curves, the amount of pepsin/pepsinogen present in each effusion was then re-calculated.

After recalculation of the total pepsin/pepsinogen content of the effusions it was observed that an over calculation had been made as the N-terminal assay results and ELISA results did not agree. Some effusions which contained acidic proteinase activity (from N-terminal assay) and had measurable levels of pepsin/pepsinogen (from ELISA),

now appeared to lack pepsin/pepsinogen protein content (e.g. effusions 3 and 4, table below). However, 69% of effusions still had measurable levels of pepsin/pepsinogen protein after re-calculation (e.g. effusions 24 and 41, table below) and these levels remained significantly higher than serum pepsinogen levels. It was apparent that an assay to measure actual cross reactivity was necessary.

Effusion	Acidic proteinase activity (pH2.2) ml ⁻¹ effusion	Pepsin/pepsinogen protein ml ⁻¹ effusion	Pepsin/pepsinogen protein ml ⁻¹ effusion after re-calculation
3	2.034	3.37	0
4	0.551	2.06	0
24	0.57	38.23	18.47
41	3.996	55.54	40.20

3.5.5.2 Measured (actual) cross reactivity

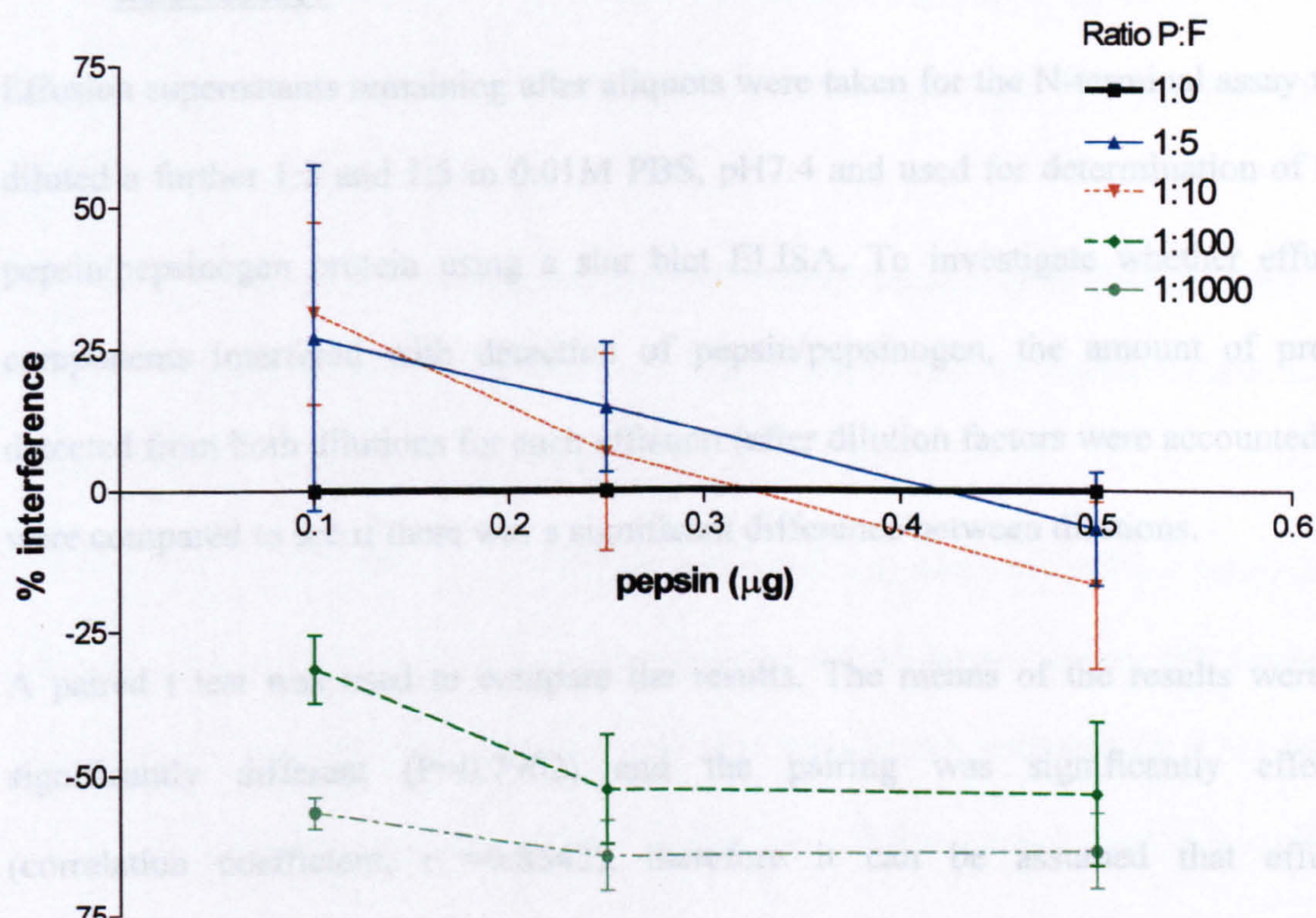
Clarification of antibody cross reactivity with fibrinogen in the pepsin ELISA was carried out as follows (n=3). Mixtures of porcine pepsin and human fibrinogen with weight:weight ratios (pepsin:fibrinogen) of 1:5, 1:10, 1:100 and 1:1000 (using 0.1, 0.25 and 0.5µg pepsin for each ratio) were blotted onto 0.2µm pore size nitrocellulose sheets and probed with the pepsin antibody in a slot blot ELISA. Antibody reactivity of each ratio was compared to that of pepsin alone and cross reactivity was evaluated.

Overall interference of fibrinogen with pepsin detection by the pepsin antibody was calculated for each weight within each ratio as shown:

$$\left[\frac{\text{Antibody binding in ratio p:f}}{\text{Antibody binding for pepsin alone}} \times 100 \right] - 100 = \text{\% Interference due to fibrinogen}$$

This ratio experiment was used to determine the amount of reactivity with the antibody that was above or below the level measured with pepsin alone and was calculated as percentage interference. With the 1:5 and 1:10 ratios, interference due to fibrinogen decreased as the amount of pepsin increased from 0.1 to 0.5 μ g. The overall interference of fibrinogen with pepsin detection (calculated as an average of all data for each ratio) decreased as the amount of fibrinogen increased from 12% (ratio 1:5) to 7% (ratio 1:10). At ratios of 1:100 and 1:1000, the interference became strongly negative at -46% and -61% respectively (Fig. 3.17). The ratio of pepsinogen to fibrinogen in serum is 1:16000 and the interference due to fibrinogen in the detection of pepsin/pepsinogen is most likely negligible therefore the pepsin/pepsinogen protein levels have not been adjusted to account for this.

Figure 3.17 Interference from fibrinogen in the pepsin ELISA.



(P) porcine pepsin, (F) human fibrinogen. P:F was a weight:weight ratio. Overall % interference for each ratio was expressed as an average of interference observed using 0.1, 0.25 and 0.5 μg pepsin. With the 1:5 and 1:10 ratios, interference due to fibrinogen decreased as the amount of pepsin increased from 0.1 to 0.5 μg . As the amount of fibrinogen present increased to 1:100 and 1:1000, the interference of fibrinogen with pepsin detection decreased.

3.5.6 Do effusion components interfere with pepsin/pepsinogen detection in the slot blot ELISA assay?

Effusion supernatants remaining after aliquots were taken for the N-terminal assay were diluted a further 1:2 and 1:5 in 0.01M PBS, pH7.4 and used for determination of total pepsin/pepsinogen protein using a slot blot ELISA. To investigate whether effusion components interfered with detection of pepsin/pepsinogen, the amount of protein detected from both dilutions for each effusion (after dilution factors were accounted for) were compared to see if there was a significant difference between dilutions.

A paired t test was used to compare the results. The means of the results were not significantly different ($P=0.7902$) and the pairing was significantly effective (correlation coefficient, $r = 0.8542$), therefore it can be assumed that effusion components do not interfere with pepsin detection in the ELISA at the dilutions used.

3.5.7 Separation of middle ear effusion components using SDS PAGE

To investigate if pepsin can be detected in effusions at the reported molecular weight after separation of the effusion using SDS PAGE, subsequent Western blotting and probing with the pepsin antibody.

Middle ear effusions (n=4) were hand homogenised separately in 2ml distilled water followed by centrifugation at 700g and 4°C for 1 hour to remove insoluble material. The supernatants were removed and freeze dried.

Two 5mgml⁻¹ solutions of porcine pepsin were used, one made in distilled water (active) and the other exposed to pH9 sodium hydroxide for 48 hours (denatured). Five-times concentrated non-reducing buffer was added to both pepsin solutions (at a 1:4 ratio of non-reducing buffer to pepsin solution), which lowered the concentration to 4mgml⁻¹.

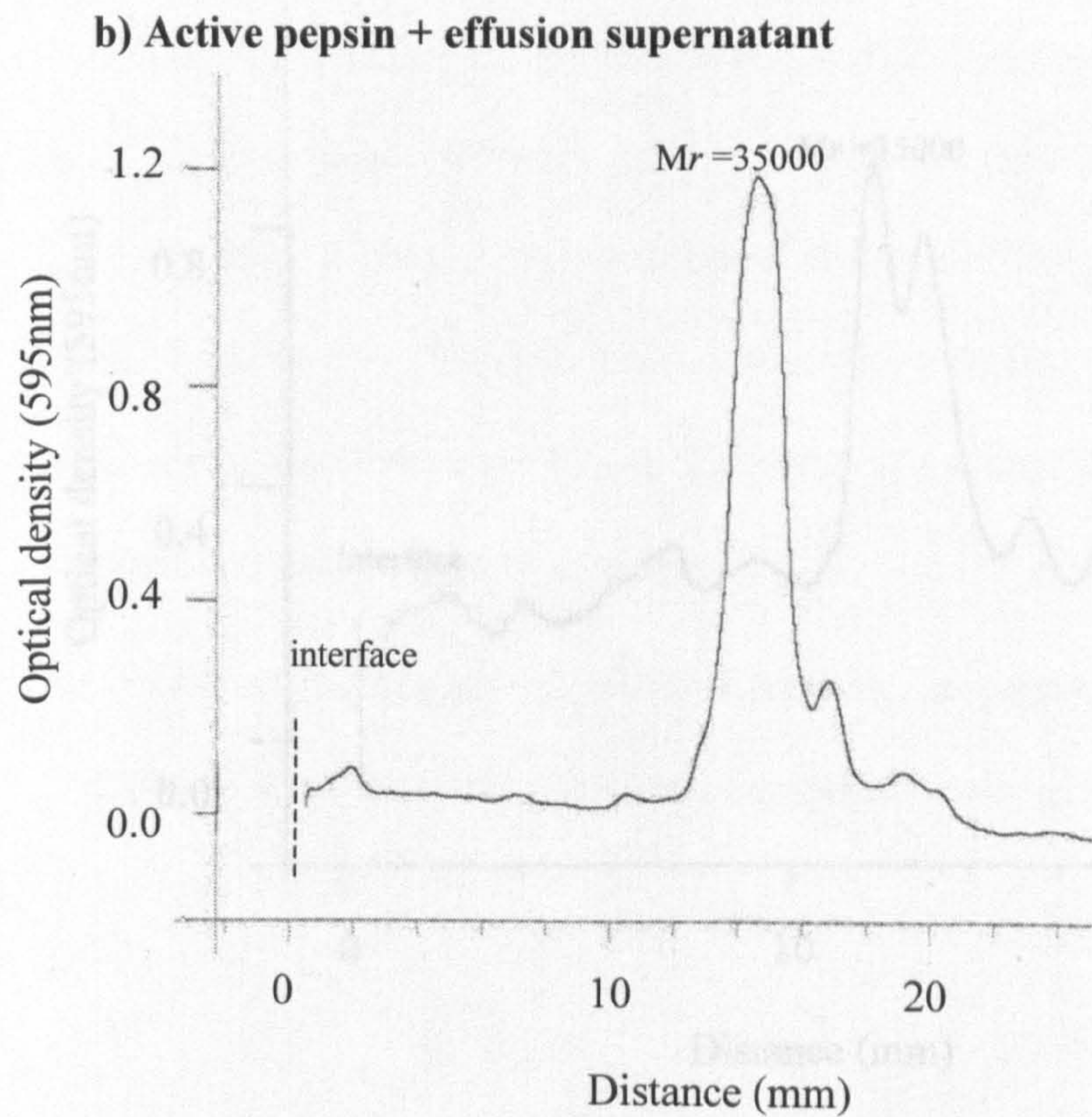
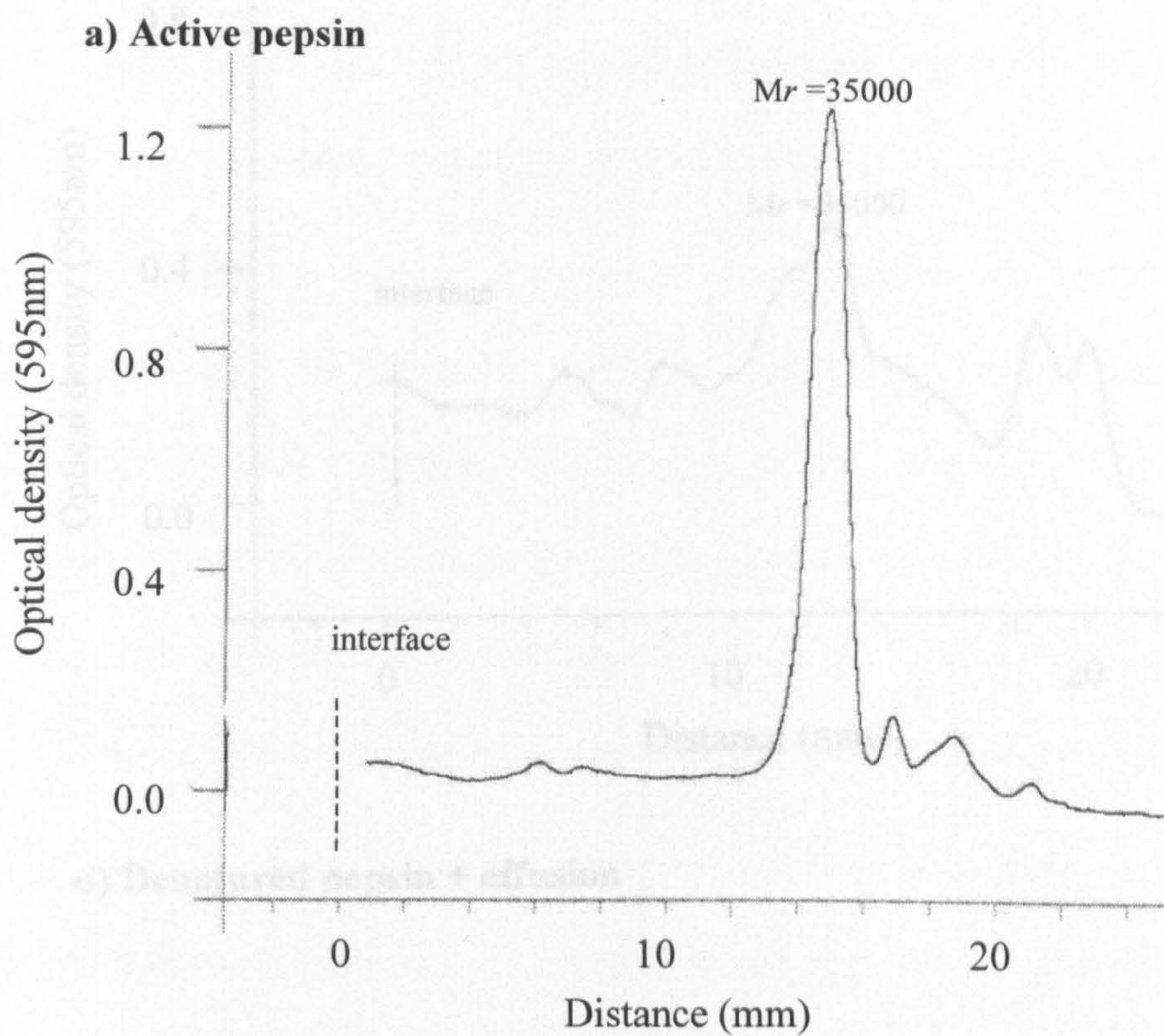
Each lyophilised effusion was solubilised in non-reducing buffer to a concentration of 10mgml⁻¹ and split into two parts. To one part was added active pepsin and to the second part was added denatured pepsin (so that 0.8µg of pepsin would be contained in 1µl).

Samples (1µl) of the solubilised effusion spiked with pepsin and the pepsin solutions alone were subjected to gel electrophoresis using 4-15% gradient polyacrylamide gels. After separation, proteins were transferred from the polyacrylamide gels to nitrocellulose membranes using a PhastTransfer Semi-dry Electrophoretic Transfer System within the PhastSystem (section 2.6.3) and the membrane was probed for the presence of pepsin using an ELISA technique (section 3.4.2). Staining was quantitated using a scanning densitometer at 595nm.

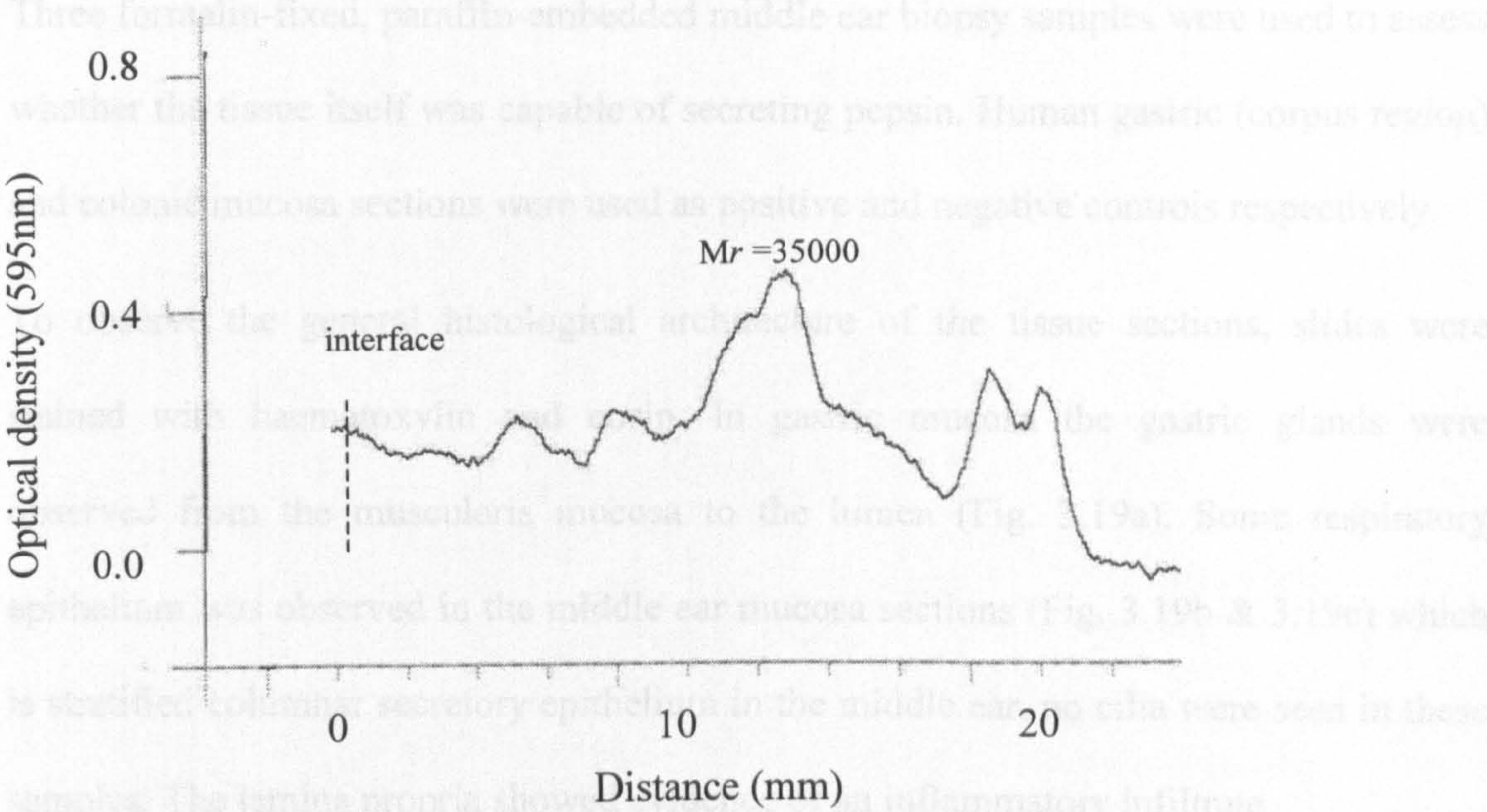
With active pepsin alone, a clear band was observed (Fig. 3.18a), 14mm from the interface and at the molecular weight for pepsin (35000). When active pepsin was run with the effusion supernatant a band was observed (Fig. 3.18b) in the same position as with pepsin alone but with a stronger intensity of staining, indicating increased antibody binding. The effusion does therefore not inhibit the movement of pepsin in SDS PAGE. With pH9 denatured pepsin there was no clear band, instead a range of low molecular weight fragments were present, still staining positive for the pepsin antibody (Fig. 3.18c). Denatured pepsin with the effusion supernatant produced a similar pattern of low molecular weight fragments as denatured pepsin alone but with a stronger intensity of staining (Fig. 3.18d). The low molecular weight material was not seen in Fig 3.18b due to a four-fold reduction in the scale in order to fit the peak on the graph.

These results demonstrate that pepsin is present in effusions but is mostly denatured, however it still reacts with the pepsin antibody.

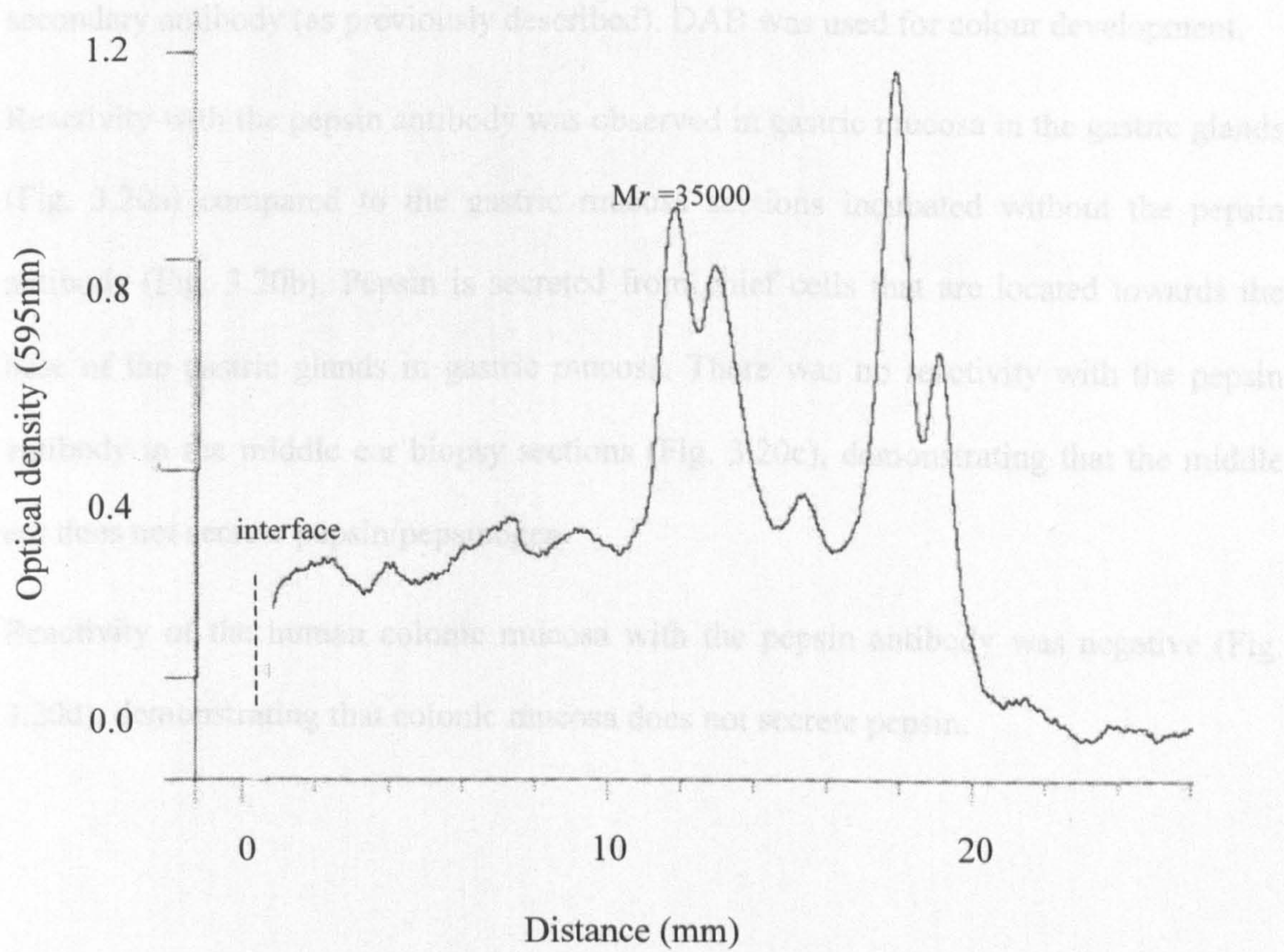
Figure 3.18 Separation of active and denatured pepsin with and without effusion material after SDS PAGE, Western blotting and probing with pepsin antibody



c) Denatured pepsin



d) Denatured pepsin + effusion



3.5.8 Immunohistochemical analysis of middle ear biopsies

Three formalin-fixed, paraffin-embedded middle ear biopsy samples were used to assess whether the tissue itself was capable of secreting pepsin. Human gastric (corpus region) and colonic mucosa sections were used as positive and negative controls respectively.

To observe the general histological architecture of the tissue sections, slides were stained with haematoxylin and eosin. In gastric mucosa the gastric glands were observed from the muscularis mucosa to the lumen (Fig. 3.19a). Some respiratory epithelium was observed in the middle ear mucosa sections (Fig. 3.19b & 3.19c) which is stratified columnar secretory epithelium in the middle ear, no cilia were seen in these samples. The lamina propria showed evidence of an inflammatory infiltrate.

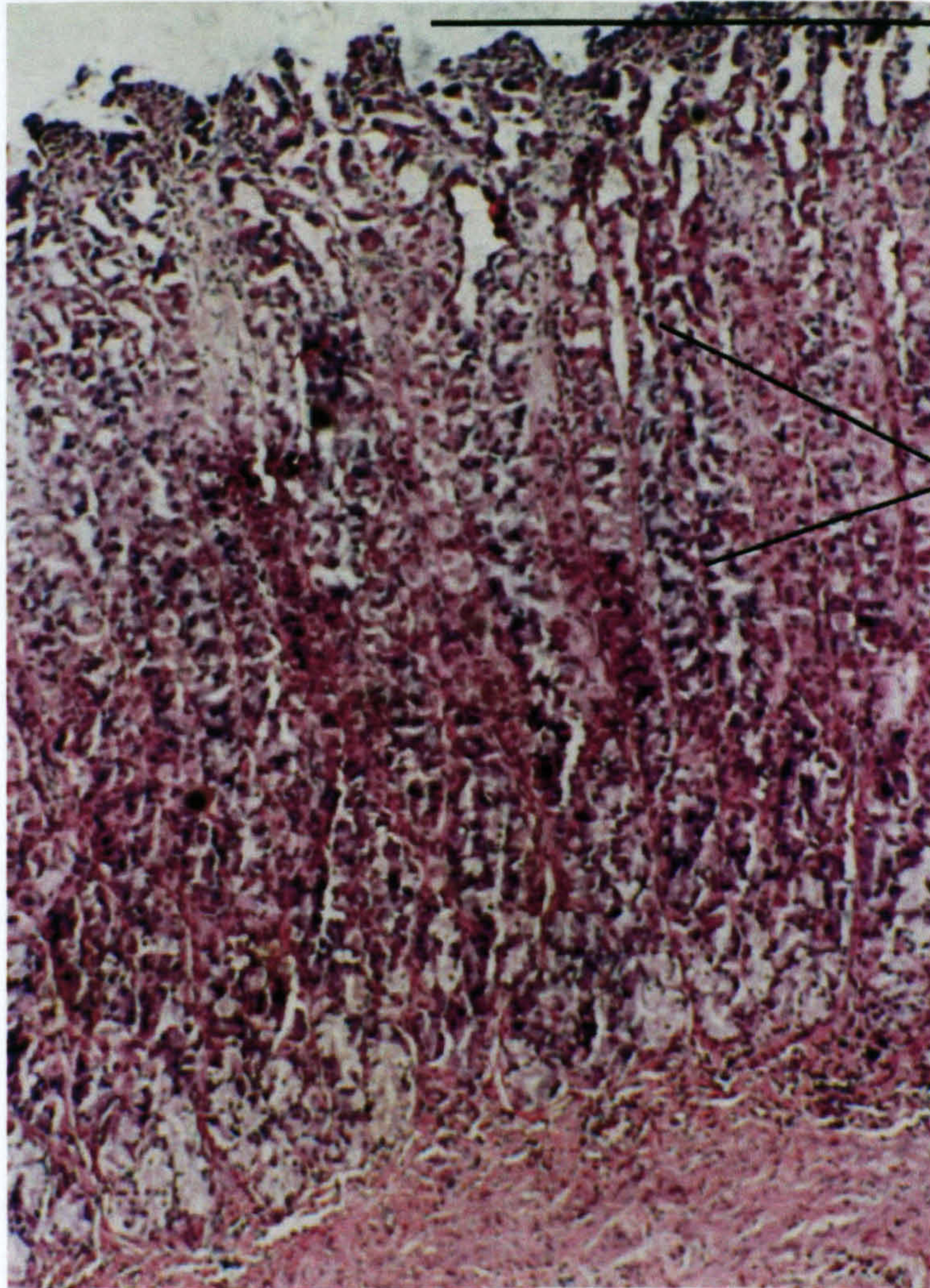
To assess the ability of the tissue to secrete pepsin, slides were incubated with the monospecific antiserum to pepsin followed by an appropriate HRP-conjugated secondary antibody (as previously described). DAB was used for colour development.

Reactivity with the pepsin antibody was observed in gastric mucosa in the gastric glands (Fig. 3.20a) compared to the gastric mucosa sections incubated without the pepsin antibody (Fig. 3.20b). Pepsin is secreted from chief cells that are located towards the base of the gastric glands in gastric mucosa. There was no reactivity with the pepsin antibody in the middle ear biopsy sections (Fig. 3.20c), demonstrating that the middle ear does not secrete pepsin/pepsinogen.

Reactivity of the human colonic mucosa with the pepsin antibody was negative (Fig. 3.20d), demonstrating that colonic mucosa does not secrete pepsin.

Figure 3.19 Photomicrographs of cryostat sections of human gastric and middle ear mucosa following haematoxylin and eosin staining

a) Human gastric mucosa (x200)

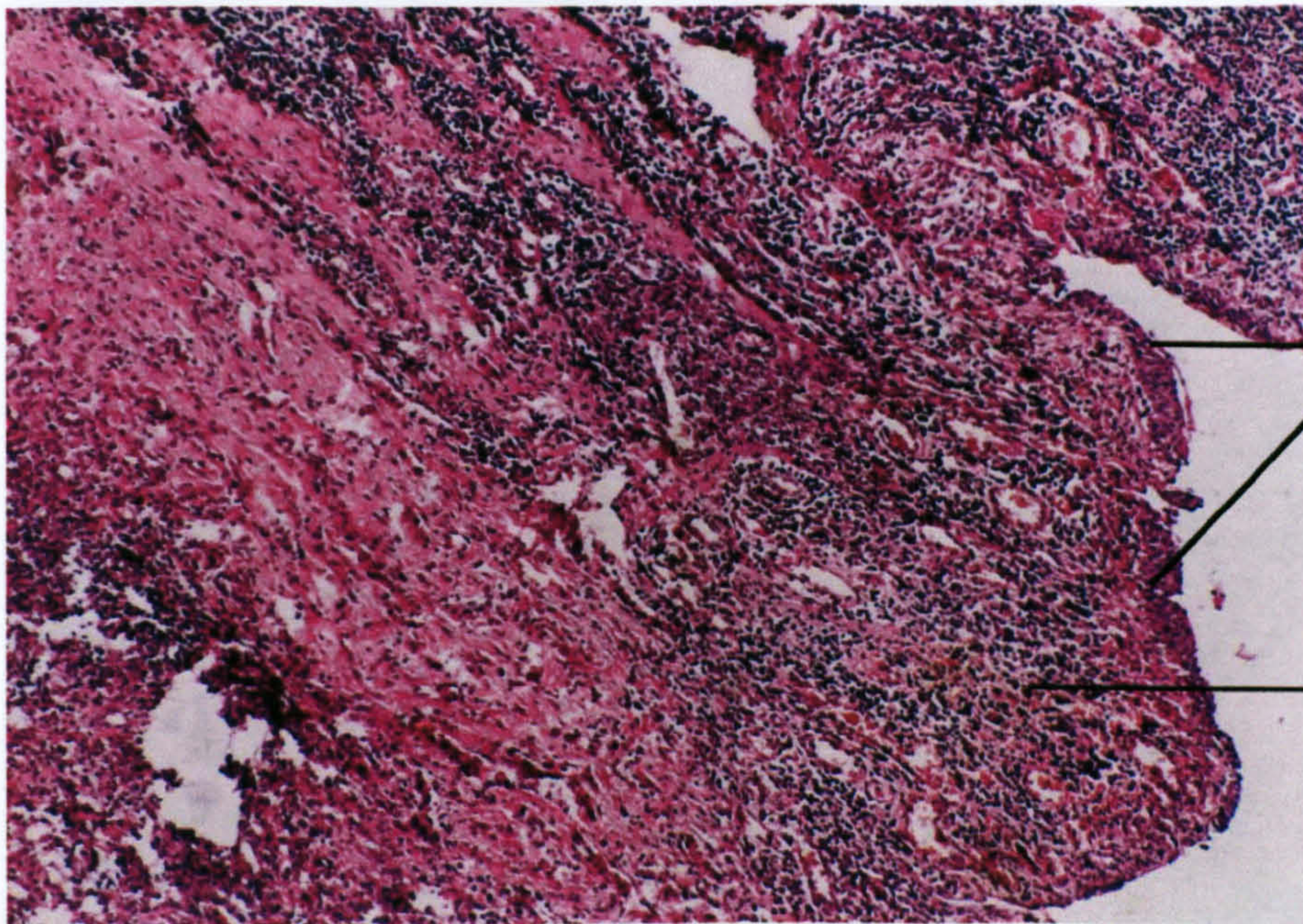


Lumen

Glandular mucosa
containing
mucus-secreting,
acid-secreting and
pepsin-secreting
cells

Muscularis
mucosa

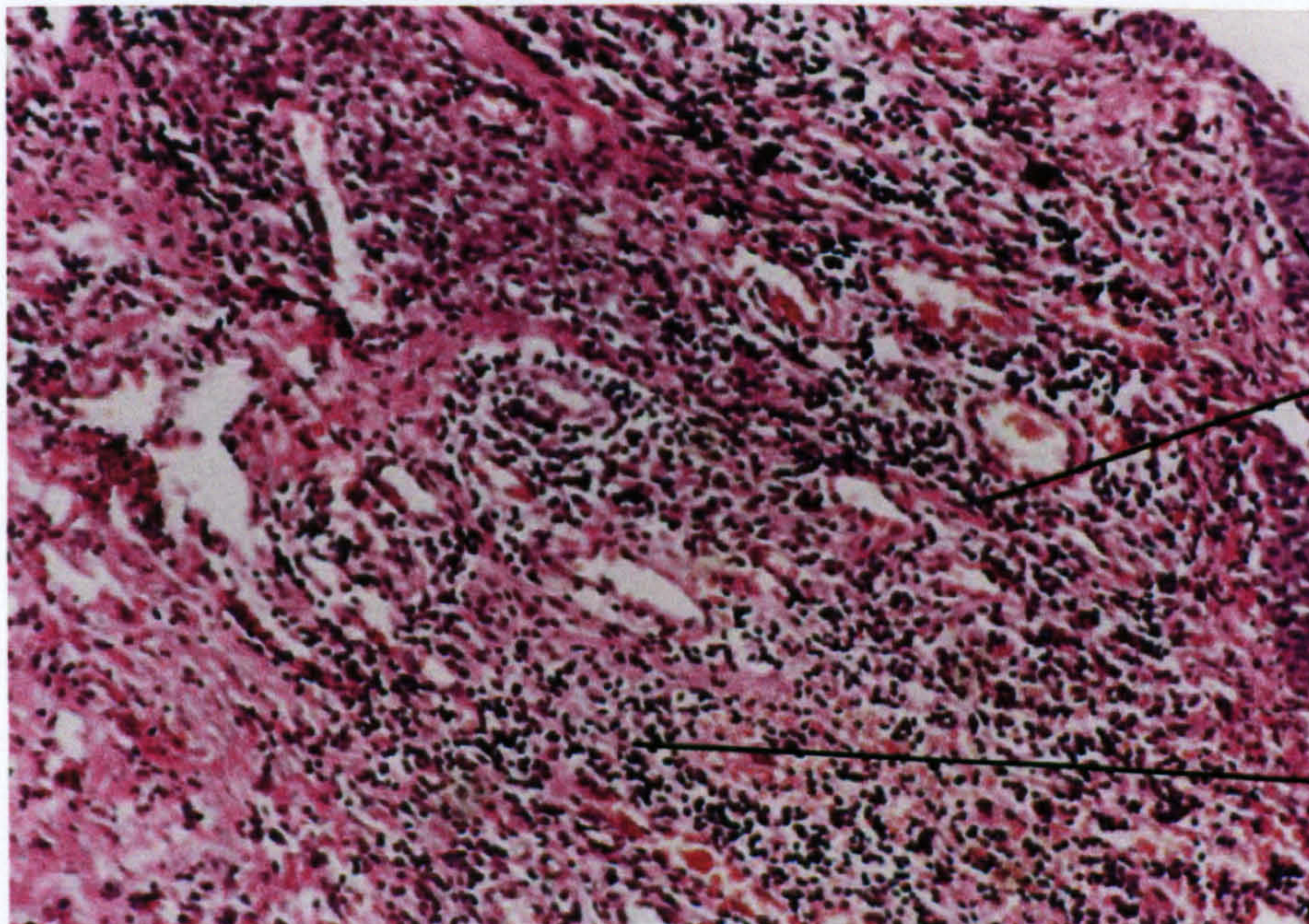
b) Human middle ear mucosa (x200)



epithelium

Inflammatory
infiltrate in
lamina propria

c) Human middle ear mucosa (x400)

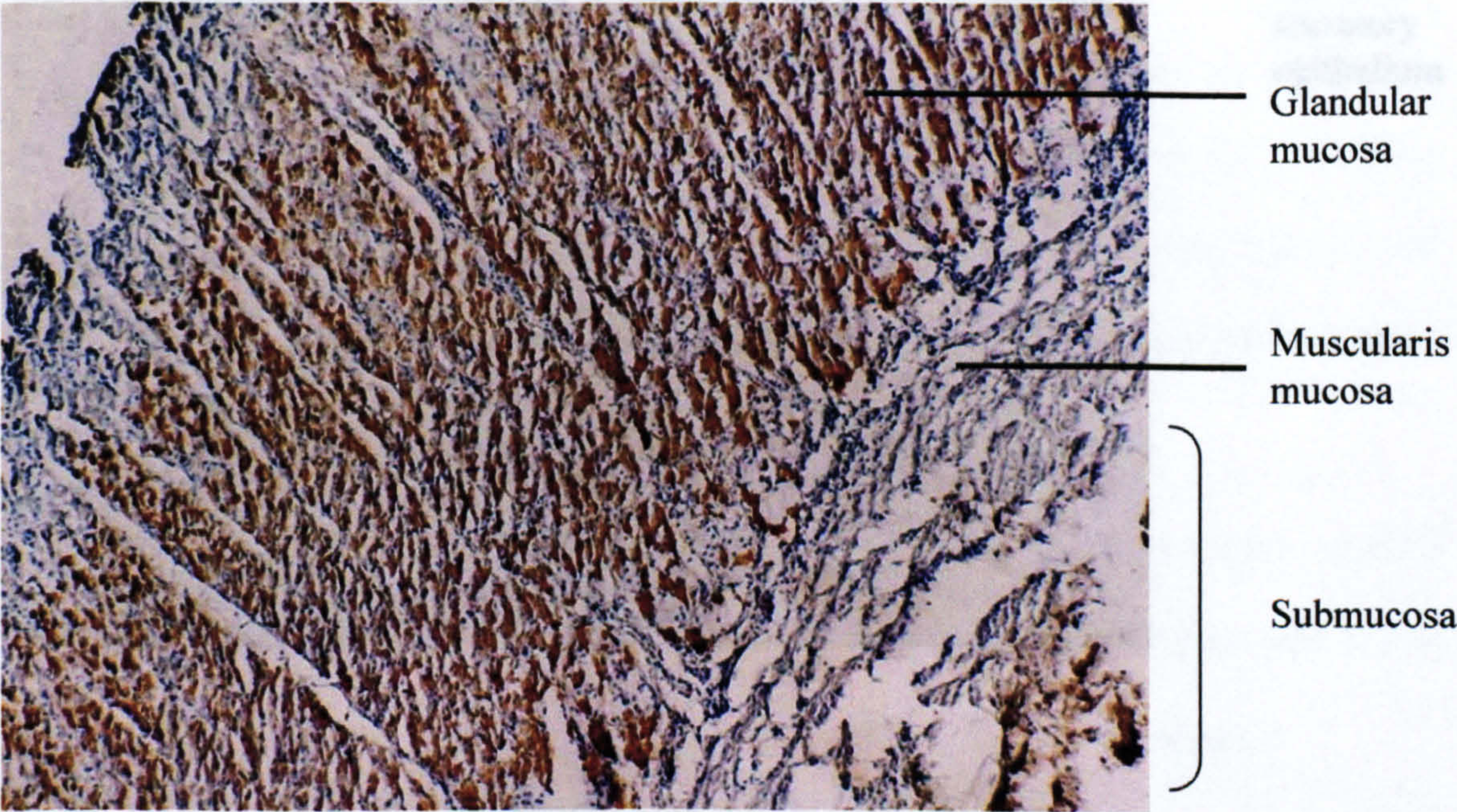


Stratified
columnar
secretory
epithelium

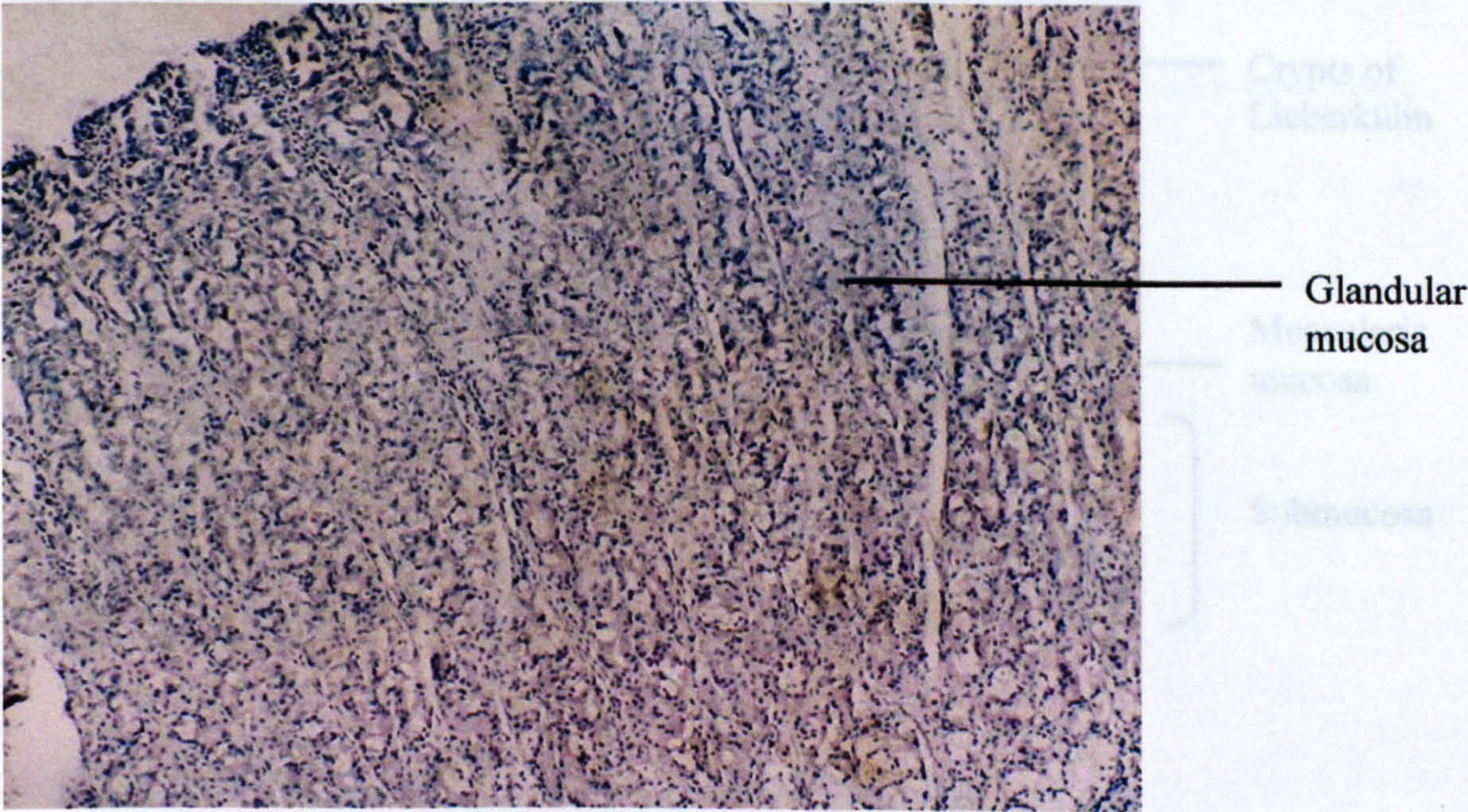
Inflammatory
infiltrate

Figure 3.20 Photomicrographs of cryostat sections of human gastric, colonic and middle ear samples following immunohistochemistry using the pepsin antibody

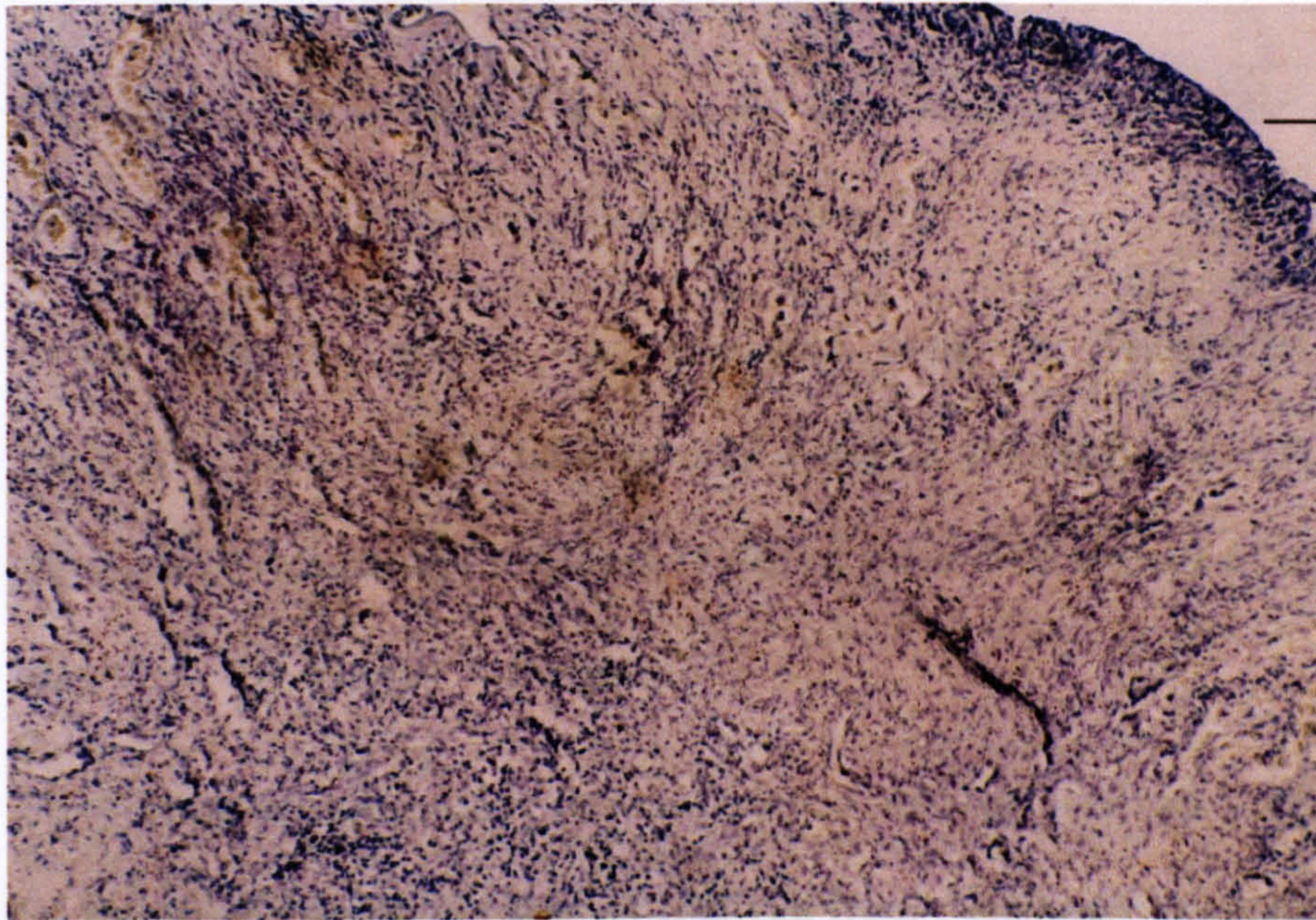
a) Human gastric mucosa (x200) with pepsin antibody



b) Human gastric mucosa (x200) without pepsin antibody

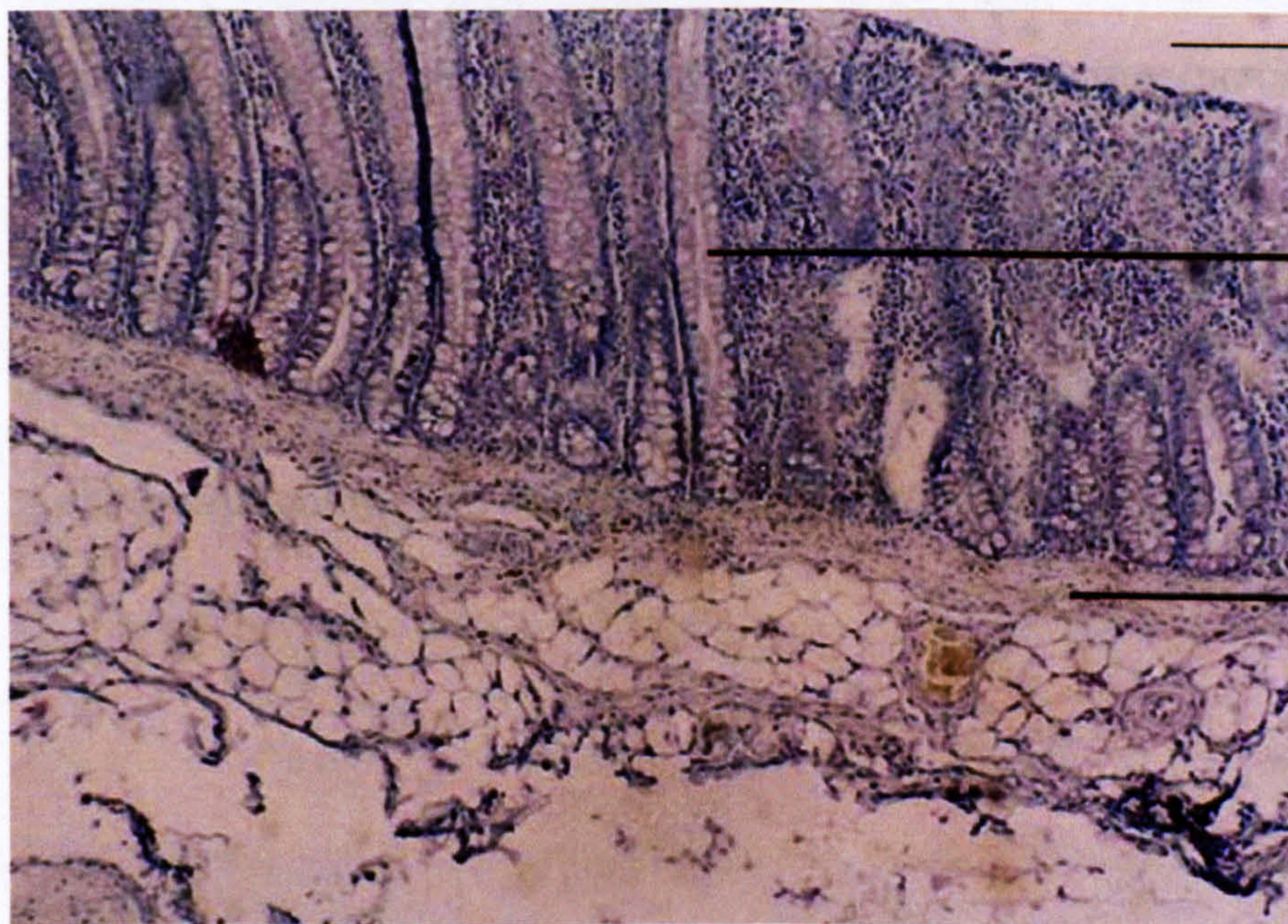


c) Human middle ear mucosa (x200) with pepsin antibody.
Unknown orientation.



Stratified
columnar
secretory
epithelium

d) Human colonic mucosa (x200) with pepsin antibody



Lumen

Crypts of
Lieberkühn

Muscularis
mucosa

Submucosa

3.6 Summary of acidic proteinase activity vs. total protein content of middle ear effusions

The supernatants of sixty-five effusions were analysed for acidic proteinase activity (section 3.2) and total protein content (section 3.4).

Acidic proteinase activity was measured using the N-terminal assay at pH2.2. Thirty-five effusions were homogenised at pH2.2. Pepsin-like activity (from pepsin and pepsinogen) was present in eleven (31%) of these. The amount of active pepsin ranged from 0.551–57.93 μgml^{-1} effusion.

Thirty effusion supernatants were exposed to pH8 buffer before being returned to pH2.2 for the assay. Pepsin like activity (derived from pepsinogen) was found in eight (27%) of these. The amount of active pepsin ranged from 3.996–147.4 μgml^{-1} effusion.

Total pepsin/pepsinogen protein present in each of the sixty-five effusions was measured using a monospecific antibody raised to pepsin (porcine stomach). The antibody could not differentiate between the two proteins. Fifty-nine out of sixty-five effusions gave a positive result with the pepsin antiserum. Pepsin/pepsinogen protein was present in the range 0.8–213.9 $\mu\text{g/ml}$ effusion (mean=43.9; SD=57.2) for the positive effusions (n=59). Interference due to fibrinogen was not taken into account.

3.7 Discussion

The present study is an investigation of the role of gastro-oesophageal reflux in OME. The aim was to analyse effusions for the presence of acidic proteinase activity, and if present, to determine the identity and source of the activity, whether from reflux of gastric contents or due to a transudate of plasma into the middle ear cleft. Pepsinogen is normally present in blood serum.

The supernatants of sixty-five effusions were tested for the presence of acidic proteinase activity (N-terminal assay) and total pepsin/pepsinogen protein content (ELISA). The majority of effusions contained pepsin/pepsinogen protein, however in only 29% of effusions was any acidic proteinase activity detected.

The pH of middle ear effusions was assessed in two ways, when measured as neat effusions (both from -20°C storage or from effusions directly after removal) using universal indicator paper the pH range was pH7–9 and when measured as a homogenate in 1ml deionised water using a pocket pH meter, the pH range was pH7.4–8.3. This is comparable to previously published data (Wezyk and Makowski, 2000) in a study of middle ear effusions from 112 children where the pH ranged from pH7–10 when measured using indicator paper. In that study, a positive correlation existed between the age of the children and the effusion pH, older children had effusions with higher pH.

The pH of effusions was so high that most pepsin would be irreversibly inhibited, explaining the high levels of pepsin/pepsinogen and denatured pepsin protein (ELISA) yet low levels of active enzyme (N-terminal assay).

The pH profiles of porcine pepsin A and pepsin from human gastric juice showed that pepsin in gastric juice could retain its activity at pH2.2 after exposure to a higher pH

(pH7.0) than porcine pepsin A alone (pH6.6) and was not completely inactivated until pH7.8 compared to pH7.1 for porcine pepsin. These pH profiles show that although most pepsin in effusions would be irreversibly inhibited, pepsin may retain some activity under these conditions and in effusions the pepsin could be protected against a pH that would normally denature the enzyme.

In both the porcine pepsin and human gastric juice pH profiles, a reduction in activity was observed with incubation at pH2.2 that was more marked in the gastric juice samples than the porcine pepsin solutions, most likely due to the much higher concentration of enzyme present. Pepsin digests protein with a pH optimum for albumin of 1.8 to 2.3 (Hirschowitz, 1984) therefore the apparent reduced activity is almost certainly due to auto digestion of the pepsin during the 20-hour pH exposure stage of the assay, as even at 4°C some activity would be expected. Porcine pepsin was used as standard in the N-terminal assay as it has been shown to have pepsin activity comparable with the major human pepsin, Pepsin 3 (Pearson *et al.*, 1986).

In a previous study (Piper and Fenton, 1965), a pH stability curve using human pepsin (homogenate of human gastric fundic mucosa) demonstrated that 100% of pepsin was stable at pH as high as pH6 (100% of peptic activity retained when measured at pH2.2). If the pH was increased above pH6, pepsin became progressively unstable until pH8 when all the enzyme was unstable and irreversibly inactivated. In the Piper study, pepsin was only exposed to pH levels in increments of 0.5 and for just ten minutes before titration back to pH2.2 and activity was measured using radio-labelled serum albumin (Klotz and Duvall, 1957).

It was noticed that incubation of human pepsin at pH7.2 at intervals of one to sixty minutes followed by titration to pH2.2 and measurement of peptic activity, produced a

similar degree of pepsin denaturation leading to the conclusion that pepsin inactivation by a suitably high pH is almost instantaneous.

Acidic proteinase activity is difficult to measure in middle ear effusions because even using the supernatant after centrifugation, the effusion components (to varying levels) mask detection of pepsin activity. An inverse correlation existed between the amount of effusion material present and the percentage of pepsin activity detected. As demonstrated by the assays in section 3.3.4, the amount of enzyme inhibition possible due to effusion material present (at levels comparable to that present in the N-terminal assay) ranged from 33-66% in the effusion pool homogenised at pH2.2 and 19-36% in the effusion pool homogenised at pH8. The extent of this range can be explained due to the two different pools of effusions used in the two studies having potentially different composition and also that pre-treatment of effusions at pH8 may reduce the protection properties of the effusions. While the effusions are exposed to pH8 there may be degradation of, or structural changes in, the components within the effusion that are responsible for masking pepsin such as protein or glycoprotein, by enzymes with a neutral pH optimum. Evidence for neutral proteinase activity in effusions has been previously demonstrated (Carrie *et al.*, 1992). Studies have shown that the pH of middle ear effusions is pH7-9, however degradation of the effusion in the middle ear does not occur, most likely because the enzymes cannot penetrate the mucoid effusion. In the current study a dilute solution of effusion is made, allowing enzyme access to all of the effusion.

A previous study has shown that mucin is the only component in effusions that determines viscosity (Carrie *et al.*, 1992) and it is most likely the presence of mucin that is inhibiting the enzyme. It has also been demonstrated that middle ear effusion

homogenates have the ability to inhibit DNase activity by up to 91% (Piezhong *et al.*, 2002). The present study shows that effusions have the potential to inhibit acidic proteinase activity and the level to which this is achieved may depend on the mucin composition of the particular effusion. High viscosity (high mucin content) effusions may have a greater ability to inhibit acidic proteinase activity than those with low viscosity (low mucin content).

It has also been demonstrated that after exposure of a known amount of pepsin (within effusion supernatants or gastric juice) to pH8, there is still a small amount (up to 6%) of acidic proteinase activity measurable at pH2.2 whereas pepsin in buffer alone is completely inactivated. This could also be due to the protective properties of the effusion and would account for the fact that some of the pH8 dialysed effusions in the N-terminal assay still had activity higher than the level for serum pepsinogen alone. A comparison of the pH profiles for gastric juice and porcine pepsin shows that gastric juice is stable at a higher pH than porcine pepsin alone by 0.5 of a pH unit. The addition of other solutes in the effusion could further stabilise the pepsin.

To advance this study, one should investigate whether the presence of both gastric juice components and middle ear effusion components protect human pepsins (in gastric juice) from complete denaturation during 20-hour storage at pH8. This could be done by adding fresh human gastric juice with known acidic proteinase activity to effusion supernatants at pH7, pH8 and pH9 and storing the samples along with effusion supernatants alone and gastric juice alone (as controls) at their respective pHs for 20 hours. After returning the samples to pH2.2, acidic proteinase activity of the effusion supernatants (with and without gastric juice) and of human gastric juice alone should be measured using the N-terminal assay to see if more proteinase activity is detected in the

gastric juice when effusion components were present and the ability of pH9 to denature pepsin.

It must not be ignored that the acidic proteinase activity detected may be in part due to other acidic proteinases apart from pepsin and the possibility of the presence of bacterial acid proteinases cannot be completely ruled out. None have been identified so far.

In the present study it was shown that the pepsin antiserum recognised both pepsin and pepsinogen and was not capable of distinguishing between the two proteins. The concentration of pepsin/pepsinogen measured in the effusions was up to 1000 times higher than the levels found in serum (reference concentrations $49.8\text{--}86.6\text{ngml}^{-1}$ (Brunner *et al.*, 1995)). Serum pepsinogen levels are well characterised and measurement of pepsinogen in adult serum in the current study fell into the normal range for adults. The level of pepsinogen in serum from children was not measured in the current study and a further study may involve analysis of both effusions and serum for pepsinogen in children. Albumin and fibrinogen levels in effusions were of the same order as those found in serum (reference concentrations $35\text{--}45\text{mgml}^{-1}$ and $2.2\text{--}4.6\text{mgml}^{-1}$ respectively). This data shows that serum factors are not being concentrated in the effusions and that the pepsin detected in middle ear effusions is unlikely to have come from a transudate of plasma. The only other sources are reflux of gastric contents or secretion of pepsin by the middle ear mucosa.

There was no cross reactivity between the pepsin antiserum and either albumin or γ -globulins however concentration dependent cross reactivity was observed with fibrinogen. Antibody binding of 20% of that for $1\mu\text{g}$ of porcine pepsin was observed for $200\mu\text{g}$ of fibrinogen. After analysis of the fibrinogen preparation using SDS PAGE it was demonstrated that no visible impurities were present at molecular weights

corresponding to those of pepsin/pepsinogen and that the antibody binding was not due to contamination of the fibrinogen with pepsin/pepsinogen. There was no significant correlation between pepsin/pepsinogen levels (measured with the pepsin antiserum) and fibrinogen levels (measured with the fibrinogen antiserum) in the same effusions, showing that the cross reactivity of fibrinogen with the pepsin antiserum was not linear (uniform).

Further measurement of the degree of cross reactivity of fibrinogen with the pepsin antibody was analysed in two ways, using a theoretical calculation and also by ratio experiments. Theoretical cross reactivity was based on the assumption that the pepsin antibody would react with all the fibrinogen and pepsin/pepsinogen. The pepsin antibody binding equivalent to the presence of fibrinogen was subtracted from the total pepsin antibody binding per effusion and the amount of pepsin/pepsinogen present in each effusion was re-calculated using the corresponding standard curves. After recalculation it was observed that the N-terminal assay results and ELISA results did not agree. Some effusions that contained acidic proteinase activity and had measurable levels of pepsin/pepsinogen protein now appeared to lack pepsin/pepsinogen protein content. However, 69% of effusions still had measurable levels of pepsin/pepsinogen protein after recalculation and these levels remained significantly higher than serum pepsinogen levels.

Measured (actual) cross reactivity experiments were used to determine the extent of the cross-reactivity using mixtures of pepsin and fibrinogen in a series of weight:weight ratios. Overall interference of fibrinogen decreased as the amount of fibrinogen present increased. At lower ratios (1:5 and 1:10 of pepsin:fibrinogen) the interference due to fibrinogen decreased as the amount of pepsin present increased, within the same ratio.

At high ratios (1:100 and 1:1000) the interference due to fibrinogen became strongly negative.

The ratio of pepsinogen to fibrinogen in human serum is 1:16000 and using the above experiments it was calculated that the amount of fibrinogen present in the effusions would not artificially raise the pepsin/pepsinogen levels measured, in fact it may lead to an underestimation and therefore the pepsin/pepsinogen data from the ELISA studies were not altered to account for interference.

SDS PAGE and Western blotting of effusion supernatants using the pepsin antibody have demonstrated that pepsin is present in effusions but is mostly in a denatured form, separating into a range of low molecular weight material with a similar pattern to pepsin that had been exposed to pH9 for 48 hours. This material was still detected by the pepsin antibody.

Haematoxylin and eosin staining of middle ear mucosa biopsy samples demonstrated that the architecture of the tissue was still sufficiently intact to be able to be used for immunohistochemistry.

Immunohistochemical analysis of biopsy samples demonstrated that the middle ear is not capable of secreting pepsin/pepsinogen as no antibody binding was detected, whereas intense staining of the gastric mucosa tissue was observed with the pepsin antibody.

The above data is strong evidence that gastro-pharyngeal reflux may have a role in OME.

To further confirm the role of reflux in OME, it would be possible to analyse effusions for the presence of other components of gastric juice not present in serum, such as gastric lipase or intrinsic factor.

Gastric lipase is a 379 amino acid glycosylated polypeptide with a pH optimum of pH3.5-7.0 (Gargouri *et al.*, 1989) It is exclusively located in chief cells of the fundic mucosa and always found together with pepsin (Moreau *et al.*, 1989). A polyclonal anti-human gastric lipase antibody has been made (personal communication, Professor R. Laugier) and it is possible that this could be used to measure gastric lipase in effusions.

Intrinsic factor is a 341 to 351 amino acid glycosylated polypeptide, secreted by the parietal cells (Hoedemaker *et al.*, 1966). The use of immunocytochemistry however, has shown it is also found in some chief cells and in the secretory ducts of salivary glands. It is possible that intrinsic factor could be measured in effusions using a rapid charcoal assay (Gottlieb *et al.*, 1965), or by ELISA using mouse anti-intrinsic factor monoclonal antibody as the primary antibody. This antibody is commercially available.

In a first reflux event, when gastric acid and pepsin are refluxed from the nasopharynx into the Eustachian tube and middle ear, the pH would be acidic. Transient damage would be caused to the Eustachian tube and the middle ear mucosa before it could be neutralised. If neutralisation only reached pH7, pepsin in the gastric juice would not be completely and irreversibly inhibited. This enzyme could then be re-activated by a second reflux event containing more acid and pepsin and cause further damage.

91% of effusions contain pepsin/pepsinogen at levels that makes the source almost certainly gastric juice. Gastric reflux may therefore be the primary factor in the

initiation of OME, instigating a cascade of inflammatory events leading to the conditions seen in the disease.

The relationship between OME and GOR is becoming increasingly important in paediatric otolaryngology however as yet there are few experimental models that demonstrate this relationship. Two recent studies (Heavner *et al.*, 2001a, Heavner, 2001 #478) have demonstrated that repeated transtympanic exposure of rat middle ear to simulated GOR causes transient abnormal Eustachian tube function, displaying an inability to appropriately rectify applied positive or negative pressure from the middle ear. An inability to equilibrate middle ear negative pressure is regarded as the primary deficit in Eustachian tube dysfunction. These researchers acknowledged the fact that transtympanic exposure to the test solution was non-physiologic and that the distal portion of the Eustachian tube had more contact than the proximal nasopharyngeal portion. A more relevant model would be one in which gastric juice components were directly placed into the nasopharynx. This would simulate GOR as it moves into the nasopharynx.

Clinicians have reported patients presenting with therapy resistant middle ear disease who, on referral to a gastroenterologist and subsequently being prescribed acid-suppressive therapy and made aware of anti-reflux measures, have had their symptoms removed (Poelmans *et al.*, 2001). In these cases the middle ear disease was thought to be associated with Eustachian tube pathological changes caused by mucosal injury from GORD.

From nasopharyngeal monitoring studies and endoscopy, it has been shown that acid, and maybe other irritative agents from the digestive tract, are able to produce or

perpetuate inflammation at every site in the larynx (Contencin *et al.*, 1995). OME and its possible infectious complications may be the outcome of GOR-induced nasopharyngeal reflux with a local chronic inflammatory process. Successful treatment of OME with anti-reflux therapy may have potential.

CHAPTER 4

PRODUCTION OF A MUC5B STANDARD FROM HUMAN WHOLE SALIVA

Chapter 4 Production of a MUC5B standard from human whole saliva

4.1 Introduction

Human saliva is a specialised, dilute aqueous secretion produced by mucous and serous cells in the three pairs of major salivary glands (parotid, submandibular and sublingual) and the numerous minor ones (labial, buccal, lingual and glosso palatine). The composition of saliva changes throughout the day depending on the relative contribution and composition of the constituent gland secretions. In the resting state, the visco-elastic submandibular-sublingual saliva constitutes much of the fluid in the mouth. The minor glands keep the buccal, labial and palatine epithelial tissues moist. Under stimulated conditions, parotid saliva can be up to 70% of the total saliva present (Veerman *et al.*, 1996) resulting in a less visco-elastic, more liquid-like secretion.

Mucus glycoproteins (mucins) are a major constituent of saliva and are secreted in a soluble form. They account for 7-26% of total salivary protein (Fox *et al.*, 1985). Mucins have a multifunctional role in the oral cavity: they lubricate oral surfaces, provide a protective barrier between hard and soft tissues and the external environment and aid in mastication and swallowing (Tabak, 1995). Salivary mucins also have the ability to bind to and agglutinate microbes (Scannapieco, 1994) acting as components of the innate host defence system in the oral cavity.

Biochemical studies have shown that two major mucin populations are present in human saliva: MG1 (high M_r mucins) and MG2 (low M_r mucins) (Prakobphol *et al.*, 1982). MG1 is more abundant than MG2 (0.23 and 0.13gml⁻¹ saliva, respectively) (Rayment *et al.*, 2000).

MG1 is a large polymeric glycoprotein with a molecular weight of over 10^6 Da. It is composed of 15% protein, 78% carbohydrate and 7% sulphate (Levine *et al.*, 1987). The oligosaccharide moiety is very heterogeneous, with carbohydrate chains ranging in size from 4-16 sugar residues (Loomis *et al.*, 1987). MG1 glycosylation and sulphation appear to be characteristic for the type of salivary gland (Veerman *et al.*, 1992). MG1 resembles mucins in other parts of the human body in size and conformation. *In situ* hybridisation and immunohistochemical studies have shown that *MUC5B* is expressed in the mucous acini of all salivary glands, suggesting that MUC5B represents a major fraction of MG1 (Cohen *et al.*, 1990; Nielsen *et al.*, 1996; Nielsen *et al.*, 1997). Northern blot analysis of human sublingual and submandibular glands using probes for MUC2-6 demonstrated that RNA from both glands contained very low levels of MUC4 transcripts in addition to high levels of MUC5B transcripts in RNA from sublingual gland and lower levels of MUC5B transcripts from submandibular gland. This data suggests that MUC4 may be a minor component of MG1 (Troxler *et al.*, 1997) but there is evidence for transmembrane regions in the coding sequence of MUC4, suggesting it is a membrane bound mucin (Moniaux *et al.*, 1999). It is possible that the MUC4 detected is a cleavage product rather than a secreted mucin; a similar situation to that which occurs with MUC1 in the breast (Boshell *et al.*, 1992). MUC1 is a membrane-bound mucin whose sequence includes a transmembrane domain (Gendler and Spicer, 1995). It is expressed on normal breast epithelium (Gendler *et al.*, 1990) yet a soluble form exists in bodily fluids, for example in breast milk. There is evidence to suggest that the mucin is released from the membrane by the action of a protease, rather than being actively secreted (Boshell *et al.*, 1992).

MG2 is a small, monomeric glycoprotein with a molecular weight of 1.25×10^5 Da (Biesbrock *et al.*, 1991). It is composed of 30.4% protein, 68% carbohydrate and 1.6% sulphate (Prakobphol *et al.*, 1982). It does not polymerise, existing as a single polypeptide chain, an exception among the secreted mucins. MG2 has a lower number of O-linked carbohydrate units than MG1, ranging in size from 2-7 residues (Levine *et al.*, 1987). These carbohydrate units have been structurally defined (Reddy *et al.*, 1985). At least two isoforms of MG2 can be distinguished (MG2a and MG2b) in human submandibular-sublingual saliva that differ in the amounts of fucose and sialic acid present (Ramasubbu *et al.*, 1991). MG2 has been fully sequenced and is encoded by the *MUC7* gene, localised on chromosome 4q13-q21 (Bobek *et al.*, 1996). *In situ* hybridisation has shown MUC7 expression to be present in serous cells within the submandibular and sublingual glands (Nielsen *et al.*, 1996). Evidence suggests that these structurally distinct mucins play different roles within the oral cavity. The enhanced rheological properties of the larger mucins lend themselves to coating functions whereas the smaller mucins have been shown to interact with a number of oral microorganisms in the soluble phase of saliva (Tabak, 1995).

It has been demonstrated that MUC5B is a major fraction of the high M_r salivary mucin (Desseyn *et al.*, 1997; Nielsen *et al.*, 1997; Thornton *et al.*, 1999) therefore isolation and purification of this mucin would provide a good standard for MUC5B in quantifying the glycoproteins present in middle ear effusions (Hutton *et al.*, 1998a) by ELISA.

Previous methods to isolate the high M_r mucin from saliva have relied on the use of 4-8M guanidine hydrochloride (GuHCl) (Troxler *et al.*, 1995; Mehrotra *et al.*, 1998; Wickstrom *et al.*, 2000), a chaotropic agent, as it was shown that using non-dissociative conditions, the low and high M_r mucins co-purify as they have a tendency to form

multi-molecular aggregates (Nieuw Amerongen *et al.*, 1995). GuHCl is a protein denaturant and therefore inhibits proteolysis. Treatment of isolated mucin with 4M GuHCl has been shown to lead to non-covalent aggregation (Snary *et al.*, 1974) and the mucin was found to be of higher molecular weight than mucin isolated in salt solutions. The aggregates do not form gels at glycoprotein concentrations (above 30mg/ml) similar to that found in mucus *in vivo* (Hutton *et al.*, 1983) suggesting that the mucin molecules have been altered in some way. For the above reasons, GuHCl is not involved in the isolation of high M_r mucin described in this chapter.

The aim of this study was to separate MG1 from MG2 in human whole saliva. As MUC5B is a major component of MG1, a relatively pure MUC5B preparation would be obtained.

4.2 Preparation of human salivary mucus

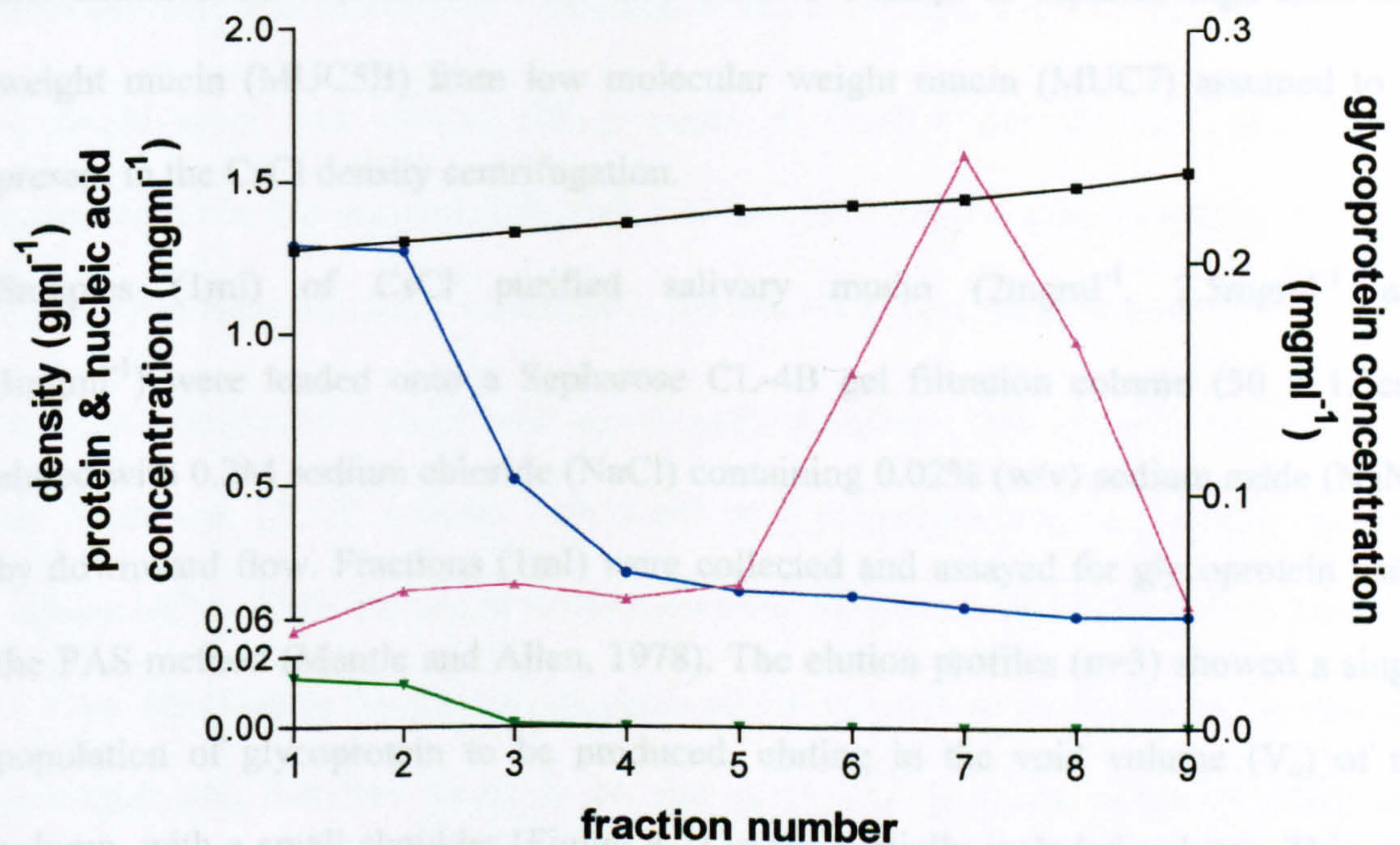
4.2.1 Purification of mucin by equilibrium density gradient ultracentrifugation in CsCl

Human whole saliva was obtained from subjects chewing on Parafilm to induce secretions. Samples were collected into an equal volume of ice cold 0.067M phosphate buffer containing protease inhibitors (Fitzgerald *et al.*, 1987). The saliva/buffer mixture was homogenised for 3-5 minutes using a Silverson mixer-emulsifier and the resulting homogenate centrifuged in an Eppendorf 5810R centrifuge at 9000rpm and 4°C for 1 hour to remove unwanted cells and debris. The supernatant was filtered through glass wool to remove particulate matter and adjusted to a density of 1.42gml⁻¹ by addition of CsCl. Following ultra centrifugation at 100,000g and 4°C for 48 hours; the resulting gradient was divided into nine equal fractions. The density of each fraction was measured followed by exhaustive dialysis against distilled water at 4°C. Aliquots of each fraction were taken for the estimation of glycoprotein, protein and nucleic acid. Glycoprotein was determined using the PAS method (Mantle and Allen, 1978) and protein and nucleic acid were estimated by optical density measurements.

A typical analysis of human whole salivary mucus (Figure 4.1) after equilibrium density ultracentrifugation in CsCl showed that there was a clear separation of glycoprotein in fractions 6-8 with density range 1.42-1.48gml⁻¹. Contaminating protein (fractions 1-5) was discarded and fraction 9 is generally unused as it is the nucleic acid-containing fraction. From the results it was observed that saliva contained little or no DNA, this is because a secretion was collected rather than being scraped from the mucosa so few cells would be present. It appears that fractions 1 to 3 contained nucleic acid, but this is

due to the protein present also having some absorbance at OD260nm. Mucin-rich fractions 6-8 were pooled, exhaustively dialysed against distilled water, freeze dried and stored at -20°C.

Figure 4.1 Analysis of human salivary mucus after equilibrium density ultracentrifugation in CsCl.



Fraction density (■) and distribution of glycoprotein (▲), protein (●) and nucleic acid (▼) are shown. Protein and nucleic acid concentration were calculated after measuring absorbance at OD280nm and OD260nm respectively then extrapolating from a nomograph based on the extinction coefficients for enolase and DNA (Warburg and Christian, 1942).

4.2.2 Isolation of purified high molecular weight mucin by gel filtration

Gel filtration on Sepharose CL-4B was used to attempt to separate high molecular weight mucin (MUC5B) from low molecular weight mucin (MUC7) assumed to be present in the CsCl density centrifugation.

Samples (1ml) of CsCl purified salivary mucin (2mgml^{-1} , 2.5mgml^{-1} and 3mgml^{-1}) were loaded onto a Sepharose CL-4B gel filtration column ($30 \times 1.5\text{cm}$) eluted with 0.2M sodium chloride (NaCl) containing 0.02% (w/v) sodium azide (NaN_3) by downward flow. Fractions (1ml) were collected and assayed for glycoprotein using the PAS method (Mantle and Allen, 1978). The elution profiles ($n=3$) showed a single population of glycoprotein to be produced, eluting in the void volume (V_0) of the column, with a small shoulder (Figure 4.2) in the partially included volume. This may be due to the two mucins not separating properly because of the short column length or overloading of the column with sample or due to *in vivo* degradation products.

To overcome this problem, Sepharose CL-2B was used which has beads with a larger pore size which would allow material eluting in the excluded volume on the Sepharose CL-4B column to be included on the CL-2B column and give two clear mucin populations. A longer column and smaller sample application were used to improve the separation characteristics.

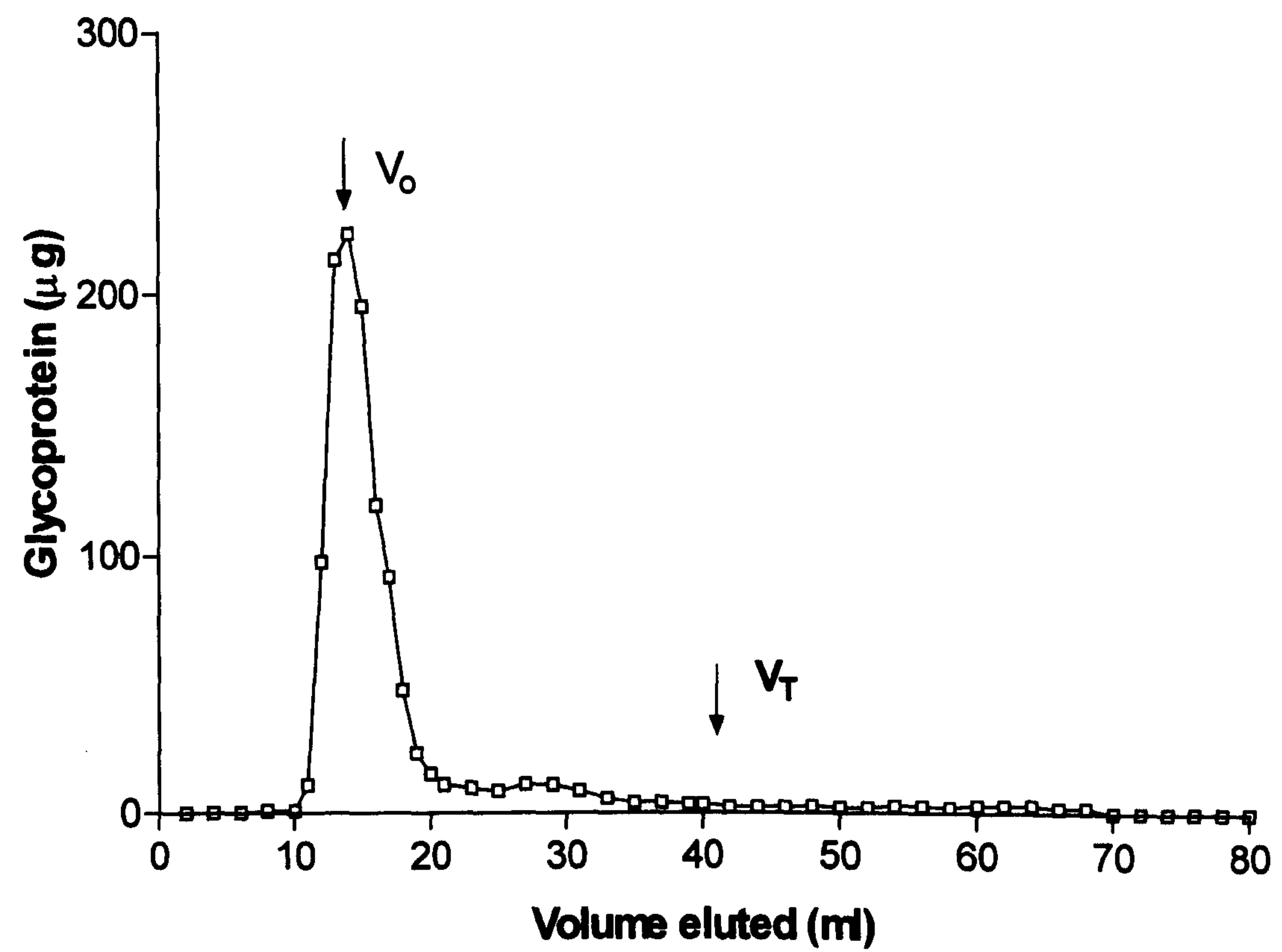
Samples (2ml) of mucin (1mgml^{-1}) were loaded onto a Sepharose CL-2B gel filtration column ($130 \times 1.5\text{cm}$) at 4°C . Mucin was eluted by upward flow of 0.2M NaCl containing 0.02% (w/v) NaN_3 using a peristaltic pump. Fractions (3ml) were collected and assayed for glycoprotein using the PAS method as before. This was repeated twice and on each occasion the majority of the glycoprotein eluted in the void volume of the

column, V_o (Figure 4.3). Using Sepharose CL-2B column the salivary mucins showed a greater amount of material extending into the column volume than when CL-4B was used but there was still no clear separation of the two mucin populations.

It would seem that without the presence of GuHCl, gel filtration seems to be unsuccessful in the separation of high and low molecular weight mucins as it appears that the two main mucins in saliva, MUC5B and MUC7 are being co-eluted.

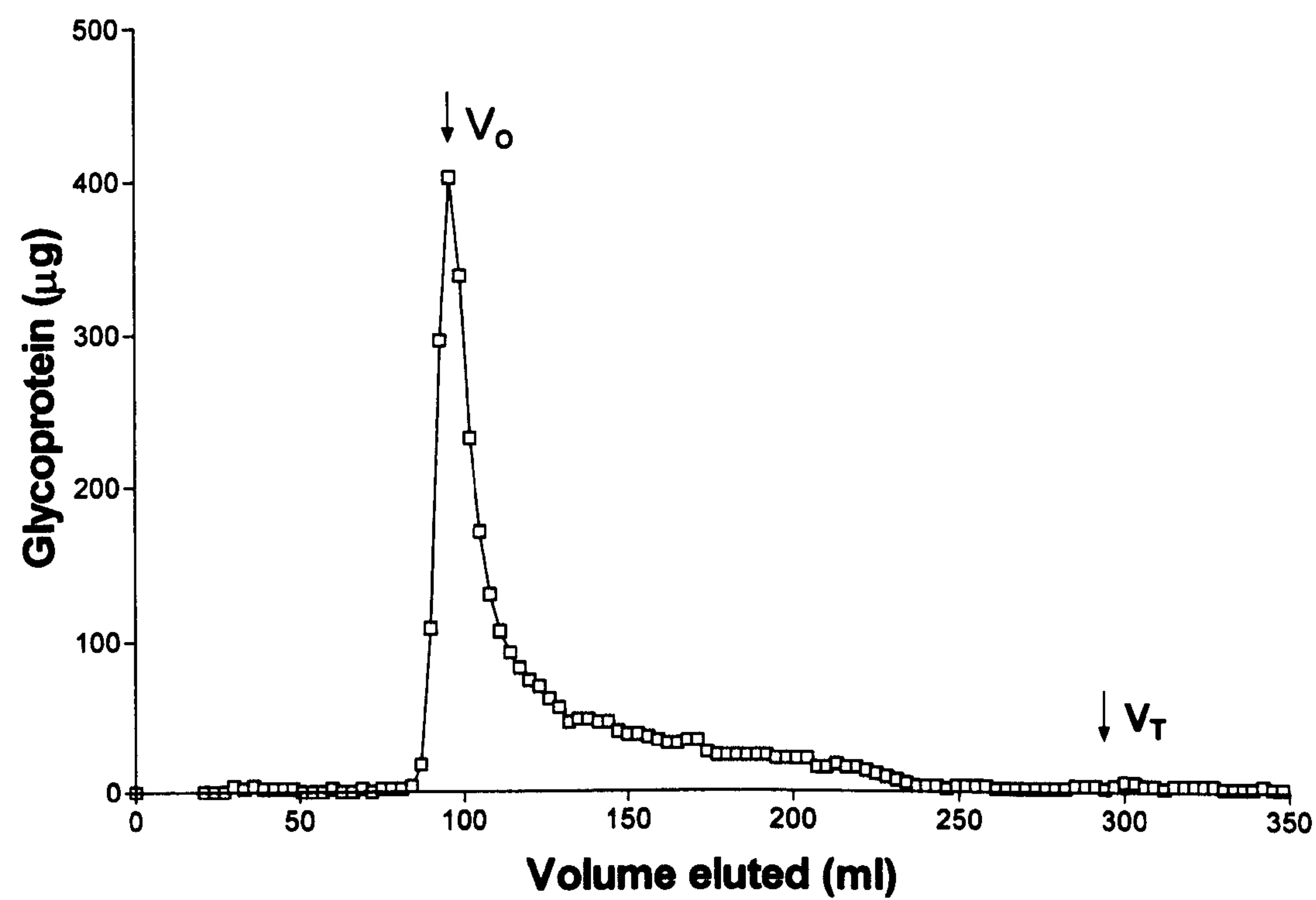
The CsCl fractionated mucin was significantly excluded (58% of eluted material) from the Sepharose CL-2B column with a tail of glycoprotein into the partially included volume (42%). The presence of lower molecular weight material is unlikely to be due to *in vitro* degradation because of inclusion of the cocktail of inhibitors in the isolation procedure and therefore likely to be due to *in vivo* products, some of which may represent degradation, present in the preparation from the collection stage.

Figure 4.2 Elution profile of 1ml of 2.5mgml^{-1} human salivary mucin (purified using CsCl density centrifugation) after gel filtration on Sepharose CL-4B.



Fractions assayed for glycoprotein using PAS method (Mantle and Allen, 1978). V_0 is the void volume of the column. V_T is the total volume of the column.

Figure 4.3 Elution profile of 2ml of 1mgml⁻¹ human salivary mucin (purified using CsCl density centrifugation) after gel filtration on Sepharose CL-2B.



Fractions assayed for glycoprotein using PAS method (Mantle and Allen, 1978). V_O is the void volume of the column. V_T is the total volume of the column.

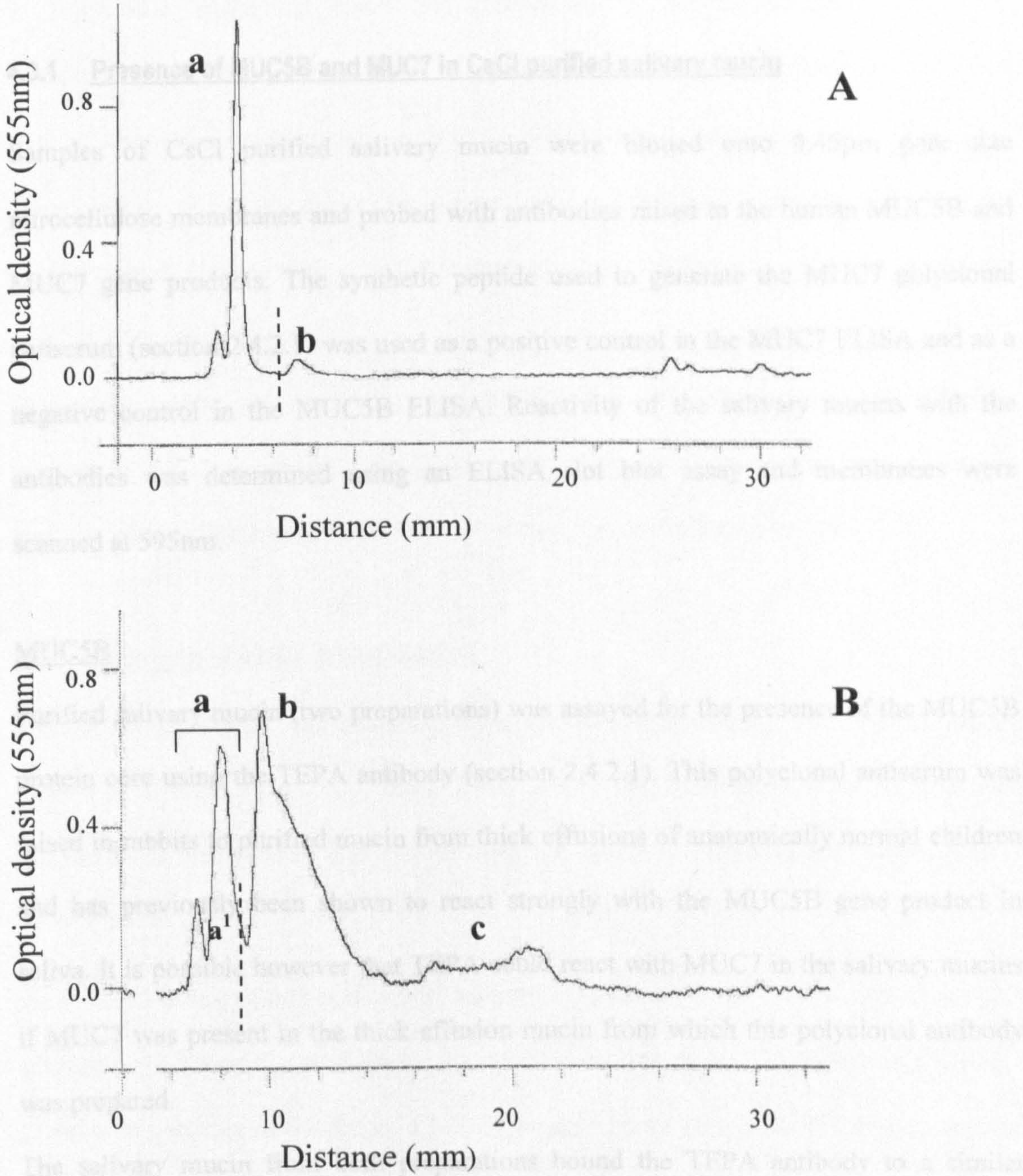
4.2.3 SDS PAGE of salivary mucin preparations

SDS PAGE was used to confirm the size and degradation state of the salivary mucin preparation. Samples (1 μ l) of salivary mucin (5mgml⁻¹ non-reducing or reducing buffer) were subjected to gel electrophoresis using a 4-15% gradient gel. Gels were stained for glycoprotein using the PAS method (Van Seuningen and Davril, 1992) and scanned at 555nm.

Following gel electrophoresis, purified salivary mucin largely remained ($86\pm1.4\%$ (n=2) of total mucin) within the stacking gel as two distinct peaks around the point of application (Figure 4.4A). All peaks within the stacking gel were denoted as band “a”. A small proportion of mucin, clearly separate from band “a” and representing lower molecular size material, peaked in the running gel adjacent to the interface of the stacking and running gel. All material within the running gel close to the interface was denoted band “b”.

Following reduction of purified salivary mucin with 5% mercaptoethanol for 2 minutes at 100°C, the proportion of material in band “a” dropped from 86% to 28%, a drop of 67%, indicating substantial breakdown of purified salivary mucin (Figure 4.4B). It was observed that some material had moved within the stacking gel (band “a¹”), demonstrating partial reduction. There was a corresponding increase (from 14% to 62%) in the proportion of low molecular size material in band “b” peaking adjacent to the interface and an appearance of lower molecular weight mucin (band “c”), 10% of the total material, extending further into the running gel than observed with the non-reduced salivary mucin preparation.

Figure 4.4 SDS PAGE of reduced and non-reduced salivary mucin.



Graph A represents purified mucin (using CsCl density centrifugation) in non-reducing buffer. Graph B represents purified mucin after reduction with mercaptoethanol (x4 expansion). Band “a”: mucin within the stacking gel, band “b”: mucin spreading into the running gel from the interface. Band “a¹”: partially reduced material within the stacking gel. Band “c”: lower molecular weight mucin extending further into the running gel than observed in the non-reduced preparation. The dashed line shows the interface of the stacking and running gel.

4.3 Antibody studies: detection of MUC5B and MUC7 in human saliva

4.3.1 Presence of MUC5B and MUC7 in CsCl purified salivary mucin

Samples of CsCl purified salivary mucin were blotted onto 0.45µm pore size nitrocellulose membranes and probed with antibodies raised to the human MUC5B and MUC7 gene products. The synthetic peptide used to generate the MUC7 polyclonal antiserum (section 2.4.2.1) was used as a positive control in the MUC7 ELISA and as a negative control in the MUC5B ELISA. Reactivity of the salivary mucins with the antibodies was determined using an ELISA slot blot assay and membranes were scanned at 595nm.

MUC5B

Purified salivary mucin (two preparations) was assayed for the presence of the MUC5B protein core using the TEPA antibody (section 2.4.2.1). This polyclonal antiserum was raised in rabbits to purified mucin from thick effusions of anatomically normal children and has previously been shown to react strongly with the MUC5B gene product in saliva. It is possible however that TEPA could react with MUC7 in the salivary mucins if MUC7 was present in the thick effusion mucin from which this polyclonal antibody was prepared.

The salivary mucin from both preparations bound the TEPA antibody to a similar degree (Figure 4.5A). Saturation occurred after 0.1µg salivary mucin per well. There was no binding between the MUC7 peptide and the TEPA antibody.

MUC7

A polyclonal antiserum raised in rabbits to a peptide sequence from the C-terminal domain of the human MUC7 gene product was used to probe mucins from both salivary mucin preparations, with up to 10µg material present per well.

There was no binding by either mucin preparation to the MUC7 antiserum, however the MUC7 peptide reacted well with the antiserum (Figure 4.5B). Saturation occurred after 1µg MUC7 peptide per well.

From these results it seemed that either MUC7 was not adhering to the nitrocellulose membrane due to its relatively small size or that the mucin had a lower buoyant density and was present in a fraction that was not pooled, i.e. not in fractions 5–8.

4.3.2 Presence of MUC7 in whole saliva

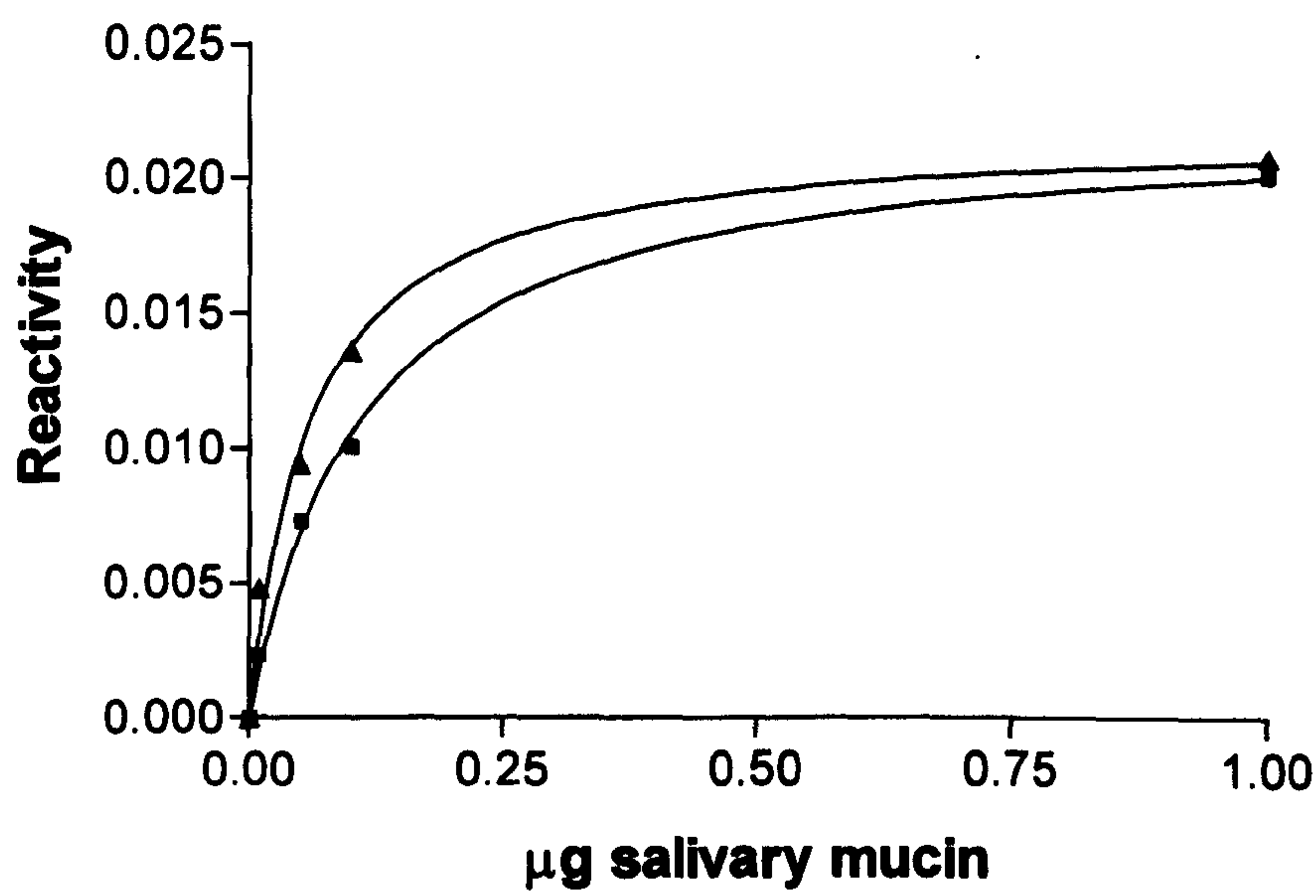
Human whole saliva was obtained from six subjects chewing Parafilm to induce secretion. Diluted (1 in 100 using PBS) whole saliva samples were blotted onto nitrocellulose membranes and probed with the antibody raised to the human MUC7 gene product.

Antibody binding was observed to varying degrees with all whole saliva samples (Figure 4.6). It was not possible to quantify the amount of MUC7 present in the saliva of each subject due to the use of a MUC7 peptide rather than a MUC7 mucin standard therefore the data are expressed as relative antibody reactivity (area under the peak from the Shimadzu scan).

MUC7 can be detected in human whole saliva but not in purified mucin from saliva therefore MUC7 must be being lost in the CsCl purification stage, possibly due to its lower buoyant density.

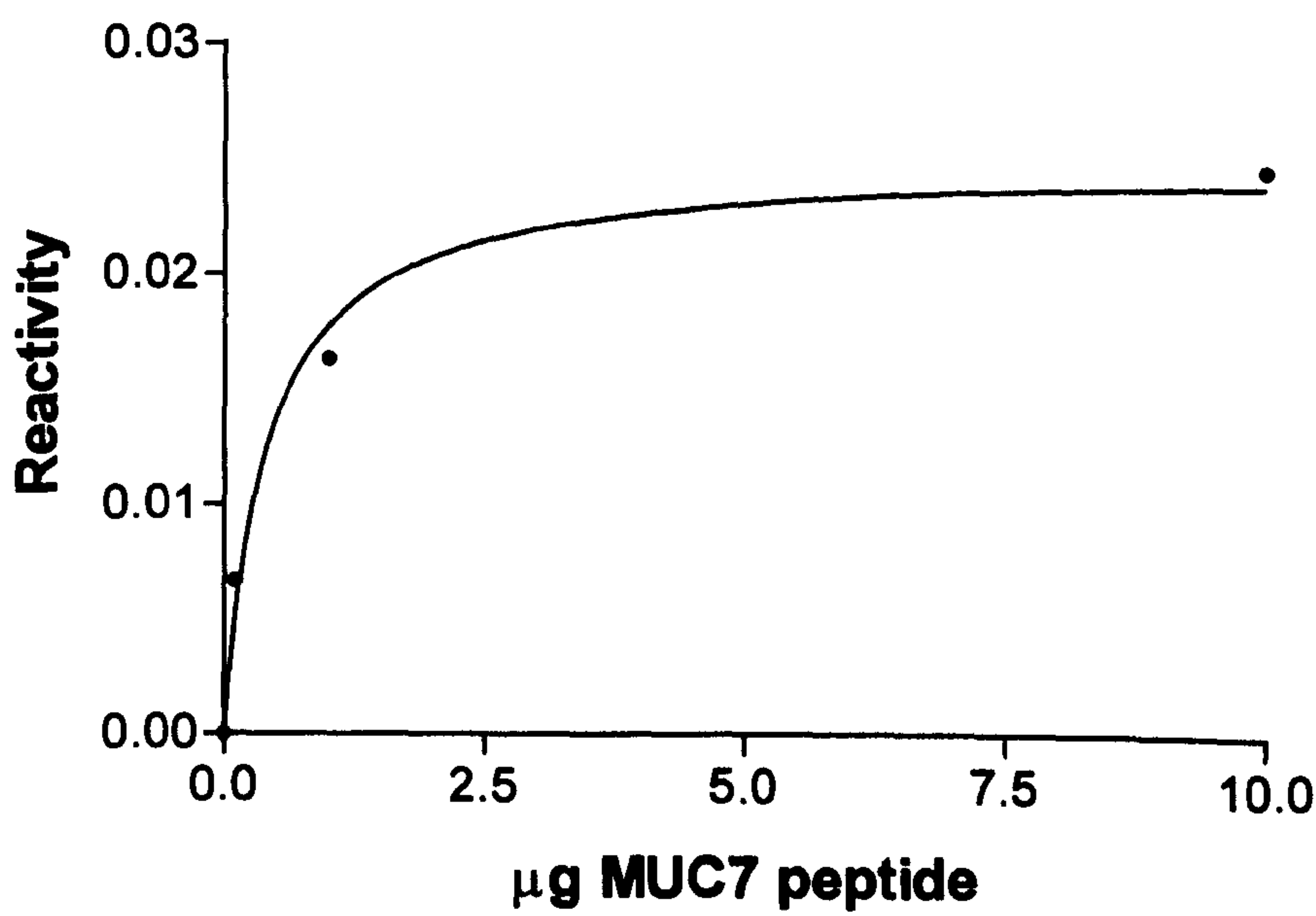
Figure 4.5

A) TEPA antibody binding to CsCl purified salivary mucin.



(▲) and (■) denote different salivary mucin preparations.

B) MUC7 polyclonal antiserum binding to MUC7 peptide



(●) denotes MUC7 peptide.

Figure 4.6 MUC7 polyclonal antibody binding to diluted whole saliva

4.3.3 Analysis of fractions from CsCl gradient

Human whole saliva was collected and purified as previously described (Section 4.1.1).

Following CsCl density gradient centrifugation, the resulting gradient was divided into

nine equal fractions of 3.9ml. The empty centrifuge tubes were washed out with a

further 3.9ml of distilled water after mild agitation. This was added as fraction 0. It

has been previously shown that MUC7 is a sticky mucin (Schier *et al.*, 1999).

fraction 0 was found to contain a small amount of material remaining in the centrifuge

tube. Fractions 1-6 were found to contain the majority of the material. Fractions 1-6 were

against distilled water as a negative control. The results are shown in Figure 4.6.

Samples of each fraction were blotted onto a nitrocellulose membrane

and probed with antibodies raised to MUC3B and MUC7 peptide products as

previously described (Section 4.3.1) in a dot blot ELISA. The MUC7 peptide and CsCl

purified salivary mucin (shown to be mainly MUC3B and containing no MUC7) were

used as positive or negative controls in each assay. Reactivity of each fraction with the

antibody was determined using an ELISA dot blot assay and membranes were

scanned at 595nm.

The results are shown in Figure 4.6.

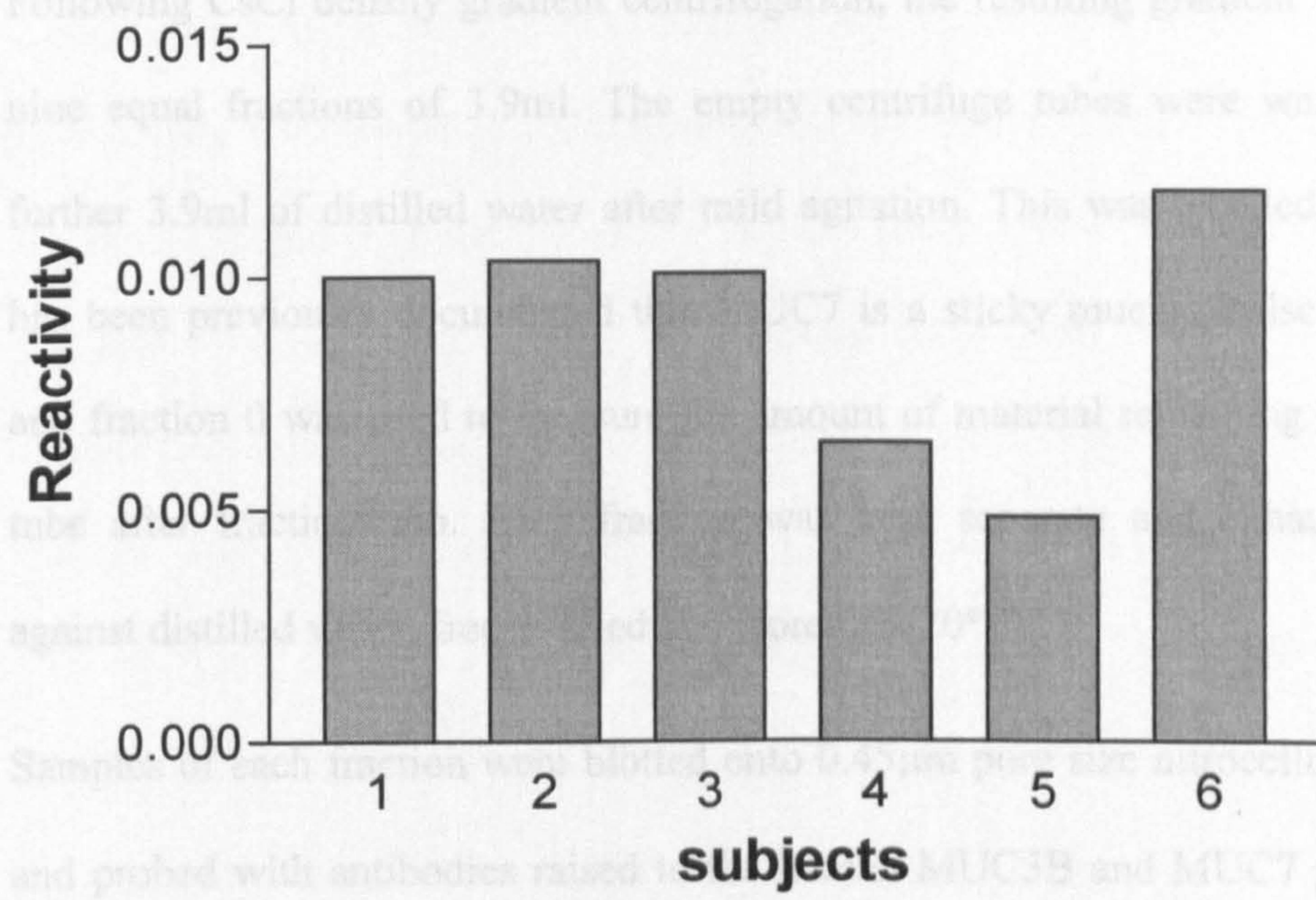
MUC3B

TEPA antibody binding was observed in all fractions 1-9 and 0 (Figure 4.7). In the

current saliva preparation most MUC3B was present in fractions 4-7. There was no

reactivity between the MUC7 peptide and the TEPA antibody (negative control), this

data is not shown.



Whole saliva was collected from six subjects, diluted in PBS and probed with the antibody raised to MUC7. Antibody binding was observed in samples from all donors.

4.3.3 Analysis of fractions from CsCl gradient

Human whole saliva was collected and purified as previously described (Section 4.1.1). Following CsCl density gradient centrifugation, the resulting gradient was divided into nine equal fractions of 3.9ml. The empty centrifuge tubes were washed out with a further 3.9ml of distilled water after mild agitation. This was labelled as fraction 0. It has been previously documented that MUC7 is a sticky mucin (Bolscher *et al.*, 1999) and fraction 0 was used to measure the amount of material remaining in the centrifuge tube after fractionation. Each fraction was kept separate and exhaustively dialysed against distilled water, freeze-dried and stored at -20°C.

Samples of each fraction were blotted onto 0.45µm pore size nitrocellulose membranes and probed with antibodies raised to the human MUC5B and MUC7 gene products as previously described (Section 4.3.1) in a slot blot ELISA. The MUC7 peptide and CsCl purified salivary mucin (shown to be mainly MUC5B and containing no MUC7) were used as positive or negative controls in each assay. Reactivity of each fraction with the antibodies was determined using an ELISA slot blot assay and membranes were scanned at 595nm.

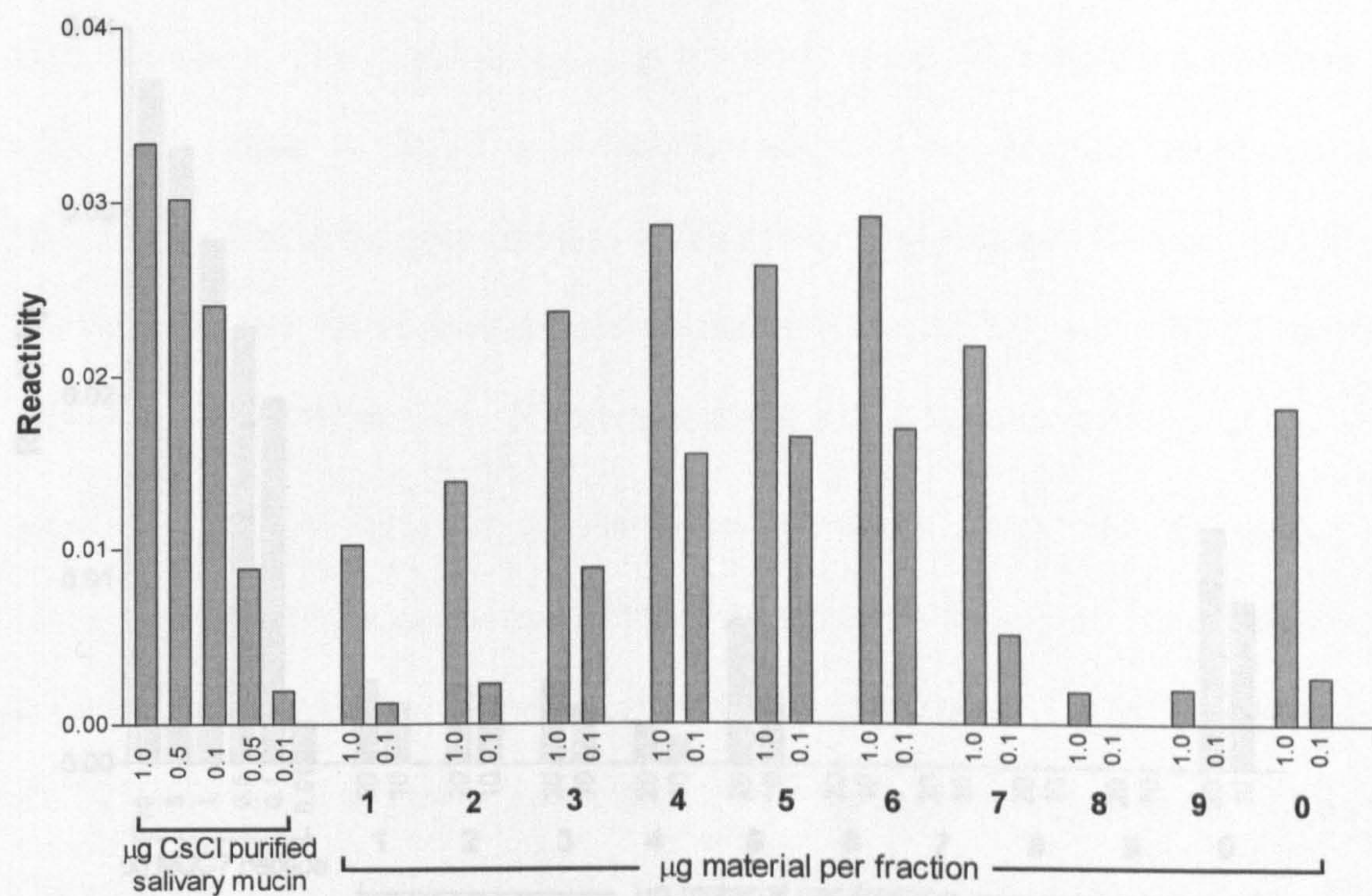
MUC5B

TEPA antibody binding was observed in all fractions 1-9 and 0 (Figure 4.7), in the current saliva preparation most MUC5B was present in fractions 4-7. There was no reactivity between the MUC7 peptide and the TEPA antibody (negative control), this data is not shown.

MUC7

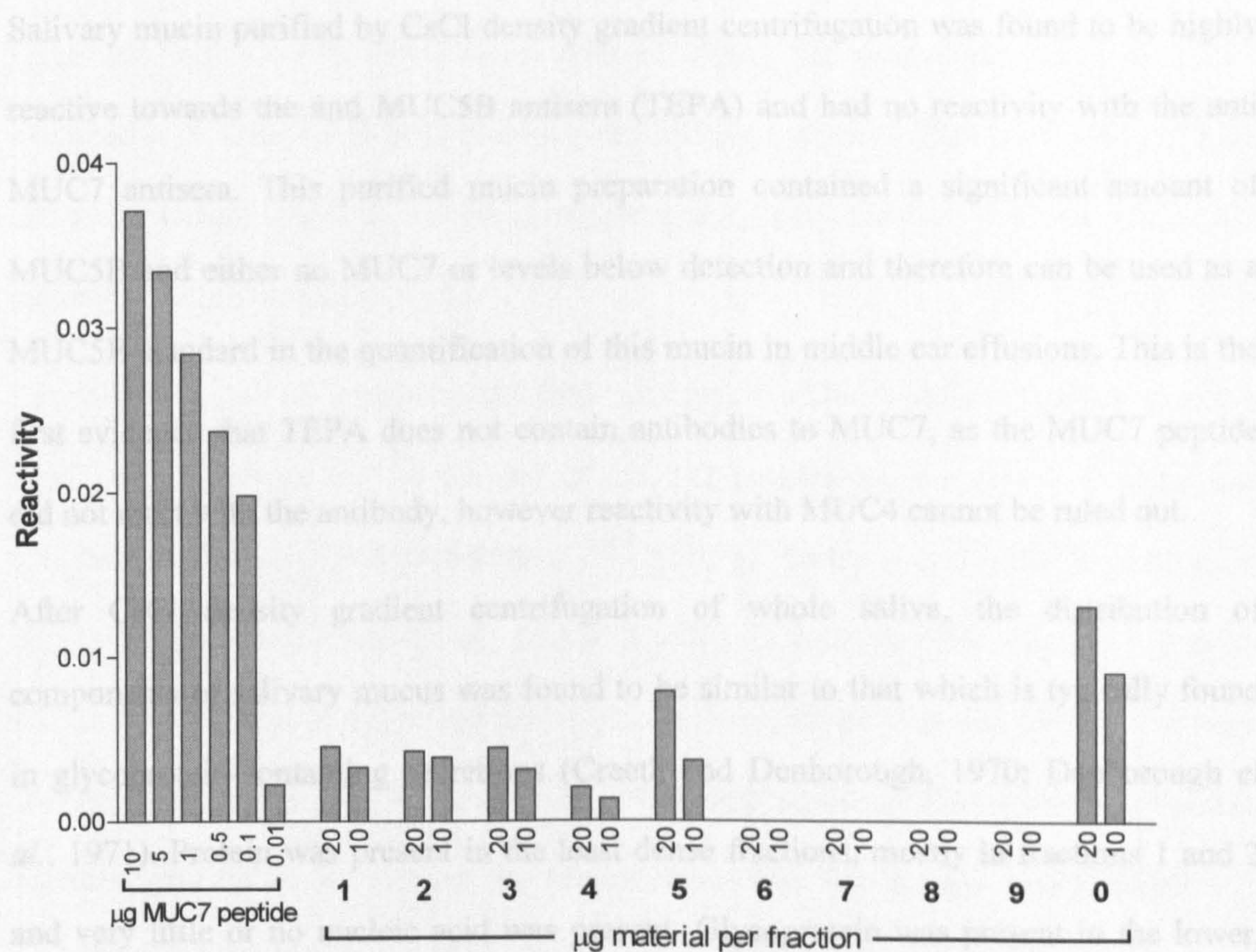
The MUC7 antiserum reacted well with the MUC7 peptide and not at all with the purified salivary mucin (negative control) (Figure 4.8). The membrane was scanned using different parameters to those in Figure 4.5, which explains the apparent difference in reactivity with the MUC7 antibody in the two assays. MUC7 mucin was detected in fractions 1-5 and 0. It was necessary to use a much greater amount of material on the slot blot to obtain reactivity with the antibody in the linear range of the standard curve against the MUC7 peptide than in the MUC5B ELISA (Figure 4.7) as it was calculated that one unit of peptide contained approximately 62.5 times more epitope than one unit of pure MUC7 (MUC7 peptide has a molecular weight of 2000 compared to the MUC7 product molecular weight of 1.25×10^5).

Figure 4.7 Distribution of MUC5B in 9 fractions after CsCl density gradient centrifugation



Samples of each fraction from CsCl density gradient were probed with MUC5B antibody in an ELISA assay and reactivity of each fraction was determined. 0.1 and 1µg of material was sufficient to obtain antibody reactivity with the MUC5B antibody (TEPA). Binding was observed in all fractions, 1-9 and 0.

Figure 4.8 Distribution of MUC7 in 9 fractions after CsCl density gradient centrifugation



Samples of each fraction from CsCl density gradient were probed with MUC7 antibody in an ELISA assay and reactivity of each fraction was determined. It was necessary to use 10 and 20µg of fraction material in the ELISA in order to obtain reactivity with the MUC7 antibody. Binding was observed in fractions 1-5 and 0.

4.4 Discussion

Salivary mucin purified by CsCl density gradient centrifugation was found to be highly reactive towards the anti MUC5B antisera (TEPA) and had no reactivity with the anti MUC7 antisera. This purified mucin preparation contained a significant amount of MUC5B and either no MUC7 or levels below detection and therefore can be used as a MUC5B standard in the quantification of this mucin in middle ear effusions. This is the first evidence that TEPA does not contain antibodies to MUC7, as the MUC7 peptide did not react with the antibody, however reactivity with MUC4 cannot be ruled out.

After CsCl density gradient centrifugation of whole saliva, the distribution of components of salivary mucus was found to be similar to that which is typically found in glycoprotein-containing secretions (Creeth and Denborough, 1970; Denborough *et al.*, 1971). Protein was present in the least dense fractions, mostly in fractions 1 and 2 and very little or no nucleic acid was present. Glycoprotein was present in the lower, more dense fractions of the CsCl gradient. To obtain a salivary mucin preparation, thought to contain both the high and low molecular weight mucins found in saliva, the glycoprotein-rich fractions were pooled.

It was expected that after gel filtration of the purified salivary mucin using a Sepharose CL-4B or CL-2B column eluted in sodium chloride azide, two distinct populations would be observed, corresponding to the MG1 (MUC5B-containing) and MG2 (MUC7-containing) mucins in saliva. Instead, a single population of mucin was consistently produced which eluted in or close to the void volume of the column. The elution profiles produced from the short Sepharose CL-4B column shall not be discussed

further due to the possibility of the column being overloaded and not having sufficient volume available for diffusion to separate the mucins.

From the Sepharose CL-2B column (130 x 1.5cm), the mucin population was 58% excluded with a tail of glycoprotein into the included volume. This is evidence of high molecular weight mucin within the preparation and also of the presence of some smaller molecular weight mucin molecules.

Previous methods of separating the high and low molecular weight mucins in saliva have generally depended on the use of gel filtration columns eluted with guanidine hydrochloride (GuHCl) (Ramasubbu *et al.*, 1991; Mehrotra *et al.*, 1998; Wickstrom *et al.*, 1998) and their results suggest that GuHCl is necessary for the separation of salivary mucin into its high and low molecular weight components. MUC7 has been labelled a 'sticky' mucin (Bolscher *et al.*, 1999) and the use of CHAPS in gel filtration columns appears to have aided the separation of the two populations. CHAPS is a zwitterionic detergent that disrupts hydrophobic interactions both within and between molecules.

Due to the tendency of multi-molecular aggregate formation to occur when GuHCl is used in the isolation of high M_r mucin and the effect this has on the properties and size of mucin molecules (Snary *et al.*, 1974; Hutton *et al.*, 1983; Hutton *et al.*, 1988), GuHCl was not used in this study. The aim was to purify MUC5B mucin from whole saliva to be used as a glycoprotein standard and therefore all attempts were made to keep the epitope to which the antibody is raised intact.

SDS PAGE gives further evidence regarding the degradation state of the mucin preparation; this has previously been validated as a method of measuring the

proportions of polymeric and degraded mucin (Rankin *et al.*, 1995). $86 \pm 1.4\%$ of the mucin was of high molecular size and remained at the point of application in the stacking gel of a 4-15% polyacrylamide gel. This is a much larger amount of high molecular size mucin than when gel filtration with Sepharose CL-2B is used. Gel filtration is based on the area available for diffusion of the molecules and there is potential for the shoulder to 'spread'. With SDS PAGE there is an absolute cut off point at a particular percentage of polyacrylamide so the material must either remain in the stacking gel at the point of application or move into the running gel.

After reduction using mercaptoethanol for 2 minutes at 100°C , there was a decrease in the purified mucin size, however 28% of the mucin still remained in the stacking gel, suggesting reduction was not complete. The change of size with reduction is evidence of a polymeric structure based on disulphide bridges, a structure previously reported for MUC5B (Loomis *et al.*, 1987). These results are similar to those obtained in a study (Kawagishi *et al.*, 1990) where purified high molecular weight mucin samples from submandibular-sublingual saliva were reduced using 0.2M mercaptoethanol for 2 hours at 37°C before electrophoresis. Glycoprotein was assayed using the PAS-Schiffs method. Non-reduced mucin all remained in the stacking gel whereas with the (reduced) mucin exposed to mercaptoethanol, the glycoprotein staining was less intense in the stacking gel and material was also present at the interface and in the running gel. The material in the running gel was defined as a 'link component' and was approximately 150,000 Da.

The results in both this study and others demonstrate partial dissociation of the mucin into its structural subunits, which is characteristic of many polymeric secreted mucins and is indicative of a breakdown of polymeric structure by cleavage of subunit

disulphide bridges in the non-glycosylated regions of the protein core. For complete dissociation of the mucin preparation into subunits, mercaptoethanol exposure time must be longer, up to 48 hours (Pearson *et al.*, 1981).

Antibody studies

Analysis of mucins present in purified salivary mucin, human whole saliva and the separate fractions of a CsCl gradient after ultra centrifugation was undertaken using the TEPA antiserum (high MUC5B specificity) and an anti-MUC7 antiserum.

The specificity of the TEPA polyclonal antiserum has been determined by immunohistochemical and papain digestion studies (Hutton *et al.*, 1998b) and almost certainly recognises a protein epitope in the non-tandem repeat region of MUC5B (Hutton *et al.*, 1998a). The anti-MUC7 polyclonal antiserum was raised to a peptide sequence at the C terminal end of the MUC7 molecule (Bobek *et al.*, 1993). It was found to react with MUC7 specifically and neither purification nor concentration of the salivary samples tested was necessary for detection of MUC7 (Bolscher *et al.*, 1999).

CsCl purified salivary mucin reacted with the anti-human MUC5B antibody, TEPA but not with the anti-human MUC7 antiserum, suggesting that the purified mucin consisted of mainly the MUC5B gene product and thus providing a significantly pure standard. It has been documented that MUC4 is also present as a minor component of the high molecular weight mucins (MG1) (Troxler *et al.*, 1997) as a very weak signal was generated by a MUC4 probe with RNA from sublingual and submandibular glands. Although there is evidence to suggest that MUC4 is a membrane-associated mucin due to the presence of a membrane-spanning hydrophobic domain (Moniaux *et al.*, 1999), it may also be present in a soluble form or as a cleavage product like MUC1 (Boshell *et*

al., 1992). In conclusion, the salivary mucin preparation may not be 100% MUC5B and this should be taken into account when measuring levels of this mucin in other samples.

Human whole saliva was found to react with the anti-MUC7 antiserum in samples from six individuals, confirming the presence of MUC7 in whole saliva and the apparent loss of this mucin during the purification stages. At present, no MUC7 standard is available, so quantification of mucin levels is not possible, only a comparison of MUC7 levels between individuals.

Analysis of the nine individual fractions and the washout fraction from the CsCl gradient showed the distribution of MUC5B to be in the fractions with higher density ($1.45\text{-}1.55\text{gml}^{-1}$) as previously demonstrated. MUC7 was detected in fractions 1-5, the lower density fractions. A significant amount of MUC7 had remained in the centrifuge tubes after fractionation as the washout fraction (fraction 0) contained a relatively large amount of MUC7. As MUC7 has been reported to be sticky, this could be the reason for the considerable amount of MUC7 remaining in the centrifuge tubes. It is possible that MUC7 is interacting with other components in the preparation such as bacterial cell wall material that would be present at the top of the gradient in fraction 1. The ionic strength in this fraction would be only 1.3gml^{-1} , which may not be enough to dissociate the mucin from other protein/lipid components. Also, potentially there are breakdown products of MUC7 present in a range of sizes that would appear higher up the gradient than the whole MUC7 molecule. A much smaller amount of MUC5B had been left in the centrifuge tubes. No cross reactivity was observed between the MUC7 peptide and the TEPA antibody showing that the TEPA antibody was recognising MUC5B and possibly MUC4 and that mucins in middle ear effusions do not contain MUC7. There was no binding between the CsCl purified salivary mucin and the MUC7 antibody

confirming that although MUC7 is present in whole saliva it is absent from the CsCl purified high density mucin preparation. In the first salivary mucin preparation (section 4.2.1), fractions 6-8 were the glycoprotein-rich fractions yet in the second preparation (section 4.3.3) the glycoprotein was mainly found in fractions 4-7. This can be explained by slight differences in the starting densities of the supernatants before caesium chloride density gradient ultra centrifugation. The glycoprotein is always present at the same buoyant density after centrifugation; however the fractions in which it is present can vary depending on the starting density of the supernatant.

MUC7 is a small, monomeric glycoprotein which is much less glycosylated than MUC5B (Levine *et al.*, 1987). The buoyant density of proteins in aqueous caesium chloride is approximately 1.3gml^{-1} and that of carbohydrate is approximately 1.6gml^{-1} (Creeth and Denborough, 1970) therefore the less glycosylated glycoprotein, MUC7 appears at a lower density than MUC5B.

It is not possible to directly compare the levels of MUC5B to MUC7 due to the relative reactivity with their respective antibodies and mucin standards are necessary. The ratio of MUC5B to MUC7 found in different studies varies depending on the collection protocols used, either human whole saliva or specific glandular secretions, this is due to the two mucin populations, MG1 and MG2 being differentially expressed by the major and minor salivary glands. If the subjects are stimulated to produce saliva by the use of acetic acid, then their salivary mucin profile may be different from saliva from subjects produced under non-stimulated conditions. It has been demonstrated that the relative contribution of each salivary gland to the total saliva present changes depending on gland stimulation (Veerman *et al.*, 1996).

In conclusion, the separation of a high molecular weight mucin from human whole saliva has been achieved using CsCl density gradient centrifugation. This mucin reacts very well with the TEPA antibody and appears to be a good, relatively pure and polymeric MUC5B standard. This will be used to quantify the amount of MUC5B gene product present in middle ear effusions of patients with otitis media with effusion.

CHAPTER 5

CHARACTERISATION AND ANALYSIS OF MIDDLE EAR EFFUSIONS

Chapter 5 Characterisation and analysis of middle ear effusions

5.1 Introduction

Middle ear effusions are viscous, mucin-rich secretions that accumulate in the middle ear cleft after a change in the mucosal epithelium from a columnar to a secretory type. This change occurs after inflammation of the middle ear, which can be caused by many factors including bacteria and their products, Eustachian tube dysfunction or by the presence of gastric refluxate. Due to their high viscosity the effusions are not cleared by the mucociliary transport system, as the cilia cannot deform the effusions in order that they be moved. Mucins are a major component of middle ear effusions (up to 32% of non-dialysable solids) and are responsible for their rheological properties (Carrie *et al.*, 1992).

Previous studies regarding the analysis of mucins in the middle ear have involved *in situ* hybridisation to assess mucin gene expression in human middle ear mucosa (transcription) and immunohistochemistry and slot blot ELISA to measure the gene products present in the tissue and in the secretion respectively (translation).

In situ hybridisation

In a study by Severn (Severn *et al.*, 1999) middle ear biopsy sections from patients undergoing middle ear surgery for OME were analysed for the expression of MUC1, MUC2, MUC3, MUC4, MUC5AC, MUC5B, MUC6 and MUC7 and all mucin genes except MUC3 and MUC6 were found to be expressed in the tissues. MUC1, MUC2, MUC4 and MUC5AC were expressed at the surface of the epithelium (MUC2 and MUC5AC in goblet cells) and were ranked (by intensity of expression and number of samples expressing the gene) as MUC4 > MUC5AC > MUC1 > MUC2. MUC5B,

MUC4 and MUC7 were expressed in the submucosal glands in the rank order MUC5B > MUC4 > MUC7. In this study, MUC4 was found to be most consistently expressed, however there is strong evidence to suggest that MUC4 is a membrane-bound mucin (Moniaux *et al.*, 1999) and it is possible that this mucin has a role in a glycocalyx that could protect the mucosa of the middle ear. Apart from MUC4, the mucin genes that were expressed in the highest intensity were MUC5B and MUC5AC, which are known to be secreted mucins (Gendler and Spicer, 1995). This study demonstrated that there was a broad range of mucin genes expressed by the inflamed middle ear mucosa and that the expression was generally related to a particular cell type as MUC5AC was found in goblet cells and MUC5B found in mucus glands.

Kawano and co-workers (Kawano *et al.*, 2000) detected MUC5B mucin gene expression only in cells located in gland-like structures in the submucosal area of middle ear mucosal specimens from patients undergoing middle ear surgery for OME. This study did not measure expression of any other mucin genes.

Lin and co-workers (Lin *et al.*, 2001) analysed middle ear tissue specimens (from subjects with and without OME) for the presence of MUC1, MUC2, MUC3, MUC5AC, MUC5B and MUC6 mucin mRNA. There is very little data regarding mucin gene expression in the normal middle ear and this study gives a comparison between the normal and diseased (inflamed) ears. In the normal tissues, only the *MUC5B* gene was expressed but in the inflamed ears expression of *MUC5B* and *MUC4* mucin genes was detected. Extensive and strong positive signals with *MUC5B* mucin antisense riboprobe and extensive positive signals with *MUC4* antisense riboprobe were demonstrated but the tissue distribution of these signals was not recorded.

There seems to be a general consensus from *in situ* hybridisation studies that in the inflamed middle ear MUC5B gene expression is detected in the submucosal glands and is at a higher level than in the normal, uninfamed ear (Lin *et al.*, 2001). These studies by Lin *et al.* and Severn *et al.* (Severn *et al.*, 1999) have demonstrated that other mucin genes are also expressed in the inflamed ear. From the ranking of levels of expression (Severn *et al.*, 1999) it can be seen that there is greater expression of the secreted mucins than the membrane associated mucins (with the exception of MUC4). It has previously been shown that tissue expression does not always correlate with the presence of the relevant gene product in the secretion (Thornton *et al.*, 1996) and this is demonstrated by a comparison of the results from *in situ* hybridisation to immunohistochemistry and slot blot ELISA studies on middle ear biopsy sections and middle ear effusions.

Immunohistochemistry

Hutton *et al.* (Hutton *et al.*, 1998b) analysed middle ear mucosa sections from surgical tissue sections for expression of MUC5B and MUC5AC using relevant antibodies and found MUC5AC protein present in goblet cells alone and MUC5B protein present only in submucosal mucus glands. A similar result was recently obtained by Chung *et al.* (Chung *et al.*, 2002) after middle ear mucosa sections were probed with a monoclonal anti-MUC5AC antibody and a positive reaction was observed in the goblet cells. These studies agree with the *in situ* hybridisation results in that the mucin genes *MUC5B* and *MUC5AC* are expressed by the inflamed middle ear mucosa and that the secreted protein products can be detected in the submucosal mucus glands and goblet cells of the mucosa.

Lin *et al.* (Lin *et al.*, 2001) demonstrated by immunohistochemistry that there was extensive expression of MUC5B and MUC4 in the inflamed middle ear epithelium compared to a weak expression of MUC5B in the normal middle ear epithelium and no other glycoprotein was detected. This data demonstrates both an upregulation in gene expression and a change in the pattern of expression.

ELISA

Purified mucin from middle ear effusions has been analysed to identify the mucins present in the secretion. To date the mucins present have only been measured in effusion pools (Hutton *et al.*, 1998a; Lin *et al.*, 2001) and never in individual secretions. In the study by Lin (Lin *et al.*, 2001), middle ear effusions were suspended in guanidine hydrochloride (GuHCl) and incubated for 24 hours with dithiothreitol (DTT), a reducing agent, and a cocktail of proteolytic inhibitors. Insoluble substances and debris were removed by centrifugation at 8000g for 20 minutes then the samples were subjected to purification by caesium chloride density gradient centrifugation and the mucin rich fractions were pooled. Mucin-rich fractions were defined as those fractions with a density of 1.35 to 1.45gml⁻¹ and a hexose to protein ratio of 2:1 or above and the mucin was probed with antibodies raised to MUC5B and MUC4 in a 96 well plate direct ELISA where the sample or standard was loaded directly onto the plate without capture by an antibody.

The MUC5B and MUC4 antibodies reacted positively with the mucin however MUC4 had only one third of the reactivity of MUC5B. These were the only two mucins measured. In this study, DTT and GuHCl were used in the isolation of the mucin and it has been shown that the use of GuHCl can lead to alterations in the mucin molecules as mucins isolated in GuHCl do not form gels at concentrations similar to those found in

mucus *in vivo* (Hutton *et al.*, 1983). DTT is a reducing agent that changes the polymeric structure of the mucin by breaking disulphide bridges between mucin molecules. This could affect the buoyant density of the mucins so that after caesium chloride density centrifugation some of the mucins may be present at a lower or higher buoyant density than the range set for the presence of mucin and therefore would not be tested.

Hutton *et al.* measured the mucin content of two pools of middle ear effusions from anatomically normal children, one pool containing effusions classified as thick (mucoid) and the other containing thin (serous) effusions. The effusions were solubilised by homogenisation in 0.067M sodium phosphate buffer, pH6.7 containing a cocktail of proteolytic inhibitors so that the mucins were present in as undegraded a state as possible. Soluble mucin was purified by two caesium chloride density gradient centrifugation stages. The purified mucins were deglycosylated and probed with antibodies raised to the non-glycosylated regions of MUC5AC, MUC5B and MUC2 and to the tandem repeat sequence of MUC1 in a slot blot ELISA.

The MUC5AC antibody recognised epitopes in mucin from both the thick and thin effusion pools but the thick effusion mucin was approximately three times more reactive than thin effusion mucin. Mucin from both effusion pools reacted with the MUC5B antibody, the thin effusion pool again had only a third of the reactivity of the thick effusion pool. No reactivity was found with either effusion pool against MUC1 or MUC2. This study suggests that MUC5AC and MUC5B are the mucins present in middle ear effusions, with MUC5B being the major secretion and MUC5AC present at much lower levels (accounting for a maximum of 15% of the effusion weight) and shows that it is important to identify the mucin gene products present in mucus secretions as well as to identify the genes expressed.

Kawano and co-workers (Kawano *et al.*, 2000) showed that an increase in MUC5B mucin mRNA transcripts had a statistically significant correlation with leukocyte numbers in the submucosa, suggesting that inflammatory cell products such as cytokines and other mediators could upregulate expression of the MUC5B mucin gene in the middle ear cleft.

Many inflammatory mediators have been identified in middle ear effusions such as arachidonic acid metabolites, histamine, surface cell adhesion molecules and chemokine RANTES (regulated upon activation, normal T cell expressed and secreted) (Jung, 1988; Skoner *et al.*, 1988; Bundo *et al.*, 1996; Schousboe *et al.*, 2001b). Several cytokines have also been measured, including IL-1 β , IL-2, IL-6, IL-8, IFN γ and TNF α (Yellon *et al.*, 1991; Himi *et al.*, 1992; Yellon *et al.*, 1992; Maxwell *et al.*, 1994; Takeuchi *et al.*, 1994; Yellon *et al.*, 1995; Pospiech *et al.*, 2000; Skotnicka and Hassman, 2000; Schousboe *et al.*, 2001a) and there is evidence to support a regulatory role of cytokines in the expression of inflammation in OME (Yellon *et al.*, 1995) via their ability to stimulate chemotaxis, immunoglobulin production, lymphocyte proliferation and other cytokine production. Cytokines have the potential to contribute to the pathology of otitis media as demonstrated by the induction of mucus hypersecretion in a rat model *in vivo* and in a human colon carcinoma-derived mucus secreting cell line *in vitro* (Lin *et al.*, 2000; Smirnova *et al.*, 2001; Smirnova *et al.*, 2002a) and could therefore participate in the production of mucin rich effusions in the middle ear cleft in OME.

The pro-inflammatory cytokines TNF- α , IL-1 β , IL-6 and IL-8 are likely to play a central role in middle ear inflammation and in the stimulation of the pathological background

of OME (Fig. 5.1), as they are present in middle ear effusions at high concentrations. The incidence and highest mean concentration values are shown in Table 5.1.

Figure 5.1 Summary of the pro-inflammatory cytokine network in middle ear inflammation (from Smirnova et al. (Smirnova *et al.*, 2002b))

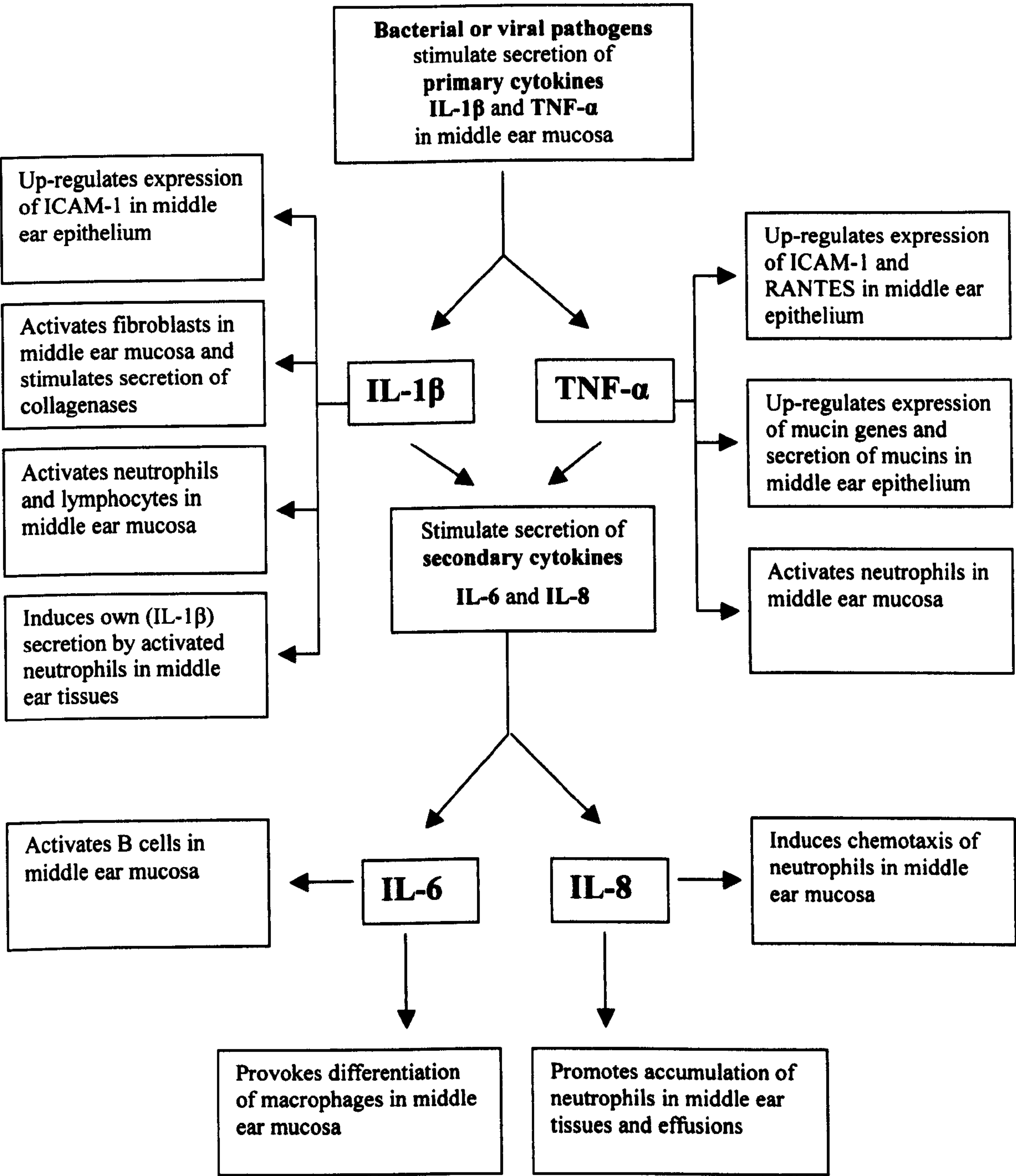


Table 5.1 Concentration of cytokines in middle ear effusions

	Concentration pgmg ⁻¹ protein	Incidence	Reference
TNFα	234.2 ± 109.1	63 – 91%	(Yellon <i>et al.</i> , 1991; Johnson <i>et al.</i> , 1997)
IL-1β	4076 ± 1510	51 – 97%	(Yellon <i>et al.</i> , 1991; Johnson <i>et al.</i> , 1997)
IL-6	173.9 ± 74.7	36 – 83%	(Yellon <i>et al.</i> , 1992)
IL-8	4805 ± 913	92 – 100%	(Maxwell <i>et al.</i> , 1994; Johnson <i>et al.</i> , 1997)

IL-6 and IL-8 are considered to be the secondary pro-inflammatory cytokines, whose secretion is stimulated by TNF- α and IL-1 β . Concentrations of IL-6 and IL-8 in middle ear effusions are shown to positively correlate with IL-1 β and TNF- α (Maxwell *et al.*, 1994; Yellon *et al.*, 1995; Skotnicka and Hassman, 2000) demonstrating the inter-connection of cytokine production during the pathogenesis of OME.

A human colon carcinoma-derived mucus secreting cell line (HT29-MTX), which produces a homogenous monolayer of goblet cells secreting MUC5AC and MUC5B and therefore is similar to the middle ear mucosa, has been used to study the effect of TNF- α , IL-6 and IL-8 on mucin gene expression in time response studies (Smirnova *et al.*, 2000; Smirnova *et al.*, 2001; Smirnova *et al.*, 2002a). The cell line was stimulated with known concentrations of human recombinant TNF- α , IL-6 or IL-8 and mucin secretion was quantitated in the culture medium using an ELISA at regular time intervals, using antibodies directed against MUC5AC and MUC5B. The results were as shown below:

Cytokine	Mucin secretion detected	Maximum secretion time	Persistence of secretion	Mucin level over control cells: MUC5AC, MUC5B
TNF- α	2 hr	7 hr	36 hr	56%, 15%
IL-6	4 hr	24 hr	72 hr	33%, 9.6%
IL-8	12 hr	72 hr	5 days	24%, 15%

The above data demonstrates that mucin secretion is induced in different ways. TNF- α induced a rapid, short-term mucin secretion, stimulation of mucin secretion by IL-6 was delayed and more prolonged and IL-8 had the most delayed and prolonged effect. These results suggest that TNF- α could be important in the first stage of the inflammatory process in the middle ear due to the high and rapid stimulation of mucin secretion. IL-6

may be responsible for maintaining the inflammation due to the lower level of mucin and prolonged mucin secretion and IL-8 may contribute to the maintenance of chronic OME by inducing prolonged mucin secretion from the goblet cell population. All cytokines induced higher levels of MUC5AC secretion than MUC5B secretion, suggesting that the mucin genes are induced in different ways and are unlikely to be controlled in the same manner.

The above studies demonstrate that in the HT29-MTX cell line, the proinflammatory cytokines upregulate mucin production, in particular MUC5AC, at different rates, for different periods of time and to different levels.

The work described in this chapter involves the biochemical and rheological analysis of individual middle ear effusions to investigate if there are correlations between effusion specific viscosity, mucin content (MUC5B and MUC5AC) and cytokine levels that are significant and can be related to the *in vitro* situation.

5.2 Analysis of the glycoprotein component of middle ear effusions

5.2.1 Middle ear effusion preparation

Serous and mucoid middle ear effusions were randomly chosen from -20°C storage, thawed at 4°C and individually hand homogenised in a known amount of ice-cold 0.067M phosphate buffer, pH6.5 containing a proteolytic inhibitor cocktail (Fitzgerald *et al.*, 1987) (section 2.3.2) before analysis. Effusion sample volumes are very small and in some cases too viscous for accurate viscosity measurements, therefore a dilution in proteolytic inhibitor buffer was necessary. The storage temperature and a proteolytic inhibitor cocktail were used to ensure the samples were exposed to minimal *in vitro* degradation by endogenous enzymes that could affect their viscosity.

5.2.2 Does the presence of proteolytic inhibitor buffer affect measurement of effusion parameters?

5.2.2.1 *Effusion non-dialysable solids*

The content of non-dialysable solids (NDS) in effusions homogenised in proteolytic inhibitor buffer (n=54) was determined by weighing the lyophilised homogenates after exhaustive dialysis at 4°C against distilled water and freeze drying (section 2.5.2). This was compared to lyophilised effusions homogenised in distilled water (n=11). The content of NDS in effusions homogenised in inhibitor buffer ranged from 0.49 to 41.3mg per effusion with a mean of 12.79 ± 1.21 (n=54) (mean \pm 1 SEM (n)). The amount of NDS in effusions homogenised in distilled water ranged from 3.9 to 29.4mg

per effusion with a mean of 14.18 ± 2.14 (n=11) (Figure 5.2). There was no significant difference in NDS content between the two groups (P=0.628, unpaired t test).

5.2.2.2 *Pig gastric mucin*

Aliquots (400 μ l) of a 1mgml⁻¹ solution of papain digested SIGMA pig gastric mucin were added to 2ml of proteolytic inhibitor buffer (n=6) or distilled water (n=4). The glycoprotein content of the lyophilised samples was determined after exhaustive dialysis at 4°C against distilled water and freeze drying, using the modified PAS method (Mantle and Allen, 1978) (section 2.4.4) with papain digested SIGMA gastric mucin as a standard.

The average amount of glycoprotein detected after exposure to proteolytic inhibitor buffer was 85.85% compared to 82.3% with glycoprotein solubilised in distilled water (data not shown). Pig gastric mucin appears to be unaffected by treatment with proteolytic inhibitors and is therefore detected in the PAS assay at a similar level to glycoprotein present in distilled water.

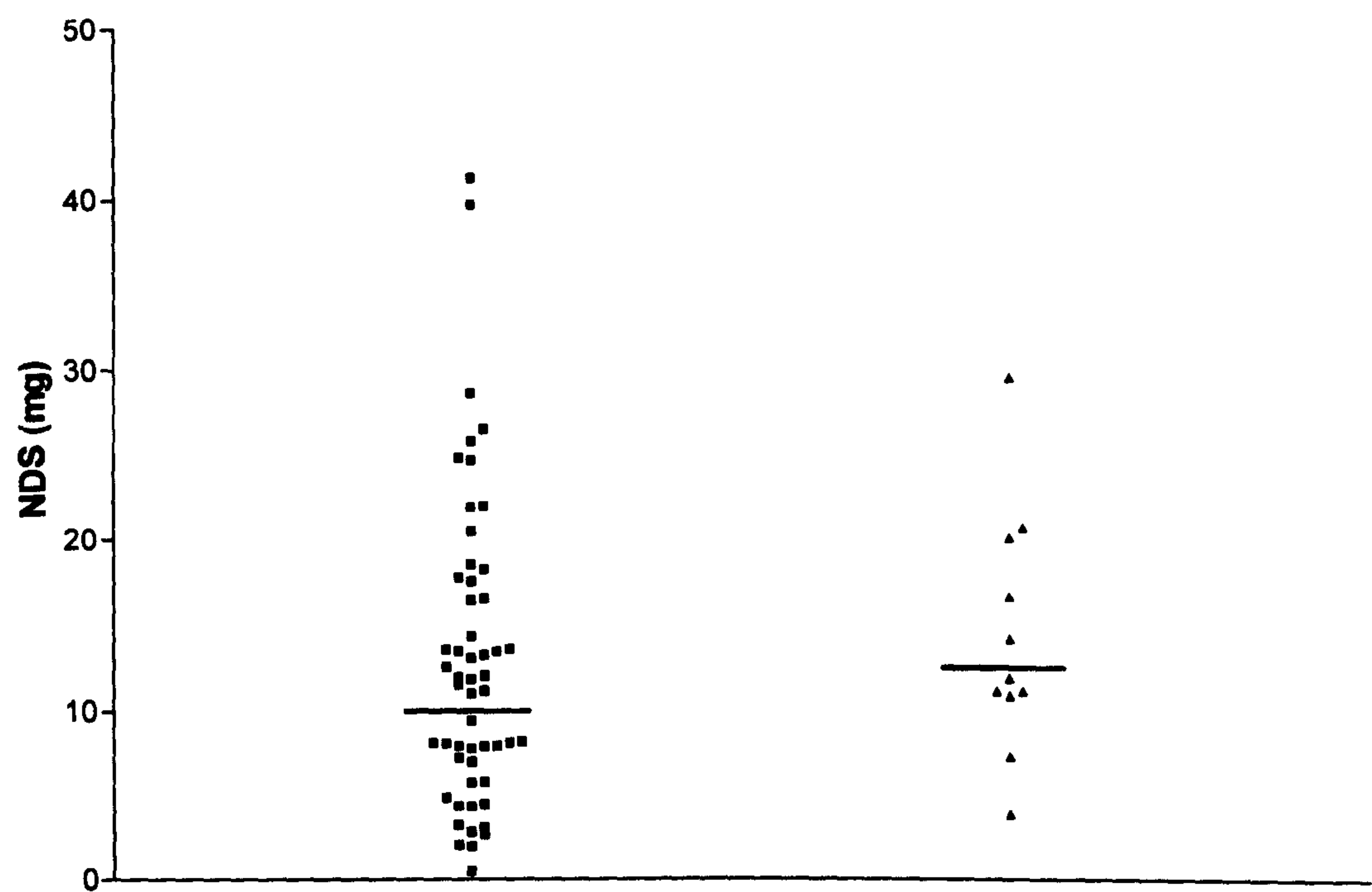
5.2.2.3 *Glycoprotein content of effusion non-dialysable solids*

The glycoprotein content of effusion NDS was estimated, after resolubilising a known amount of each effusion NDS in distilled water, by the modified PAS method (Mantle and Allen, 1978). 54 effusions were originally homogenised in proteolytic inhibitor buffer and 11 were homogenised in distilled water, the glycoprotein content of effusions in the two groups were compared.

The glycoprotein content of NDS from effusions homogenised in inhibitor buffer ranged from 2.34 to 18.76% of NDS with a mean of 5.61 ± 0.39 (n=54). The glycoprotein content of NDS from effusions homogenised in distilled water ranged from

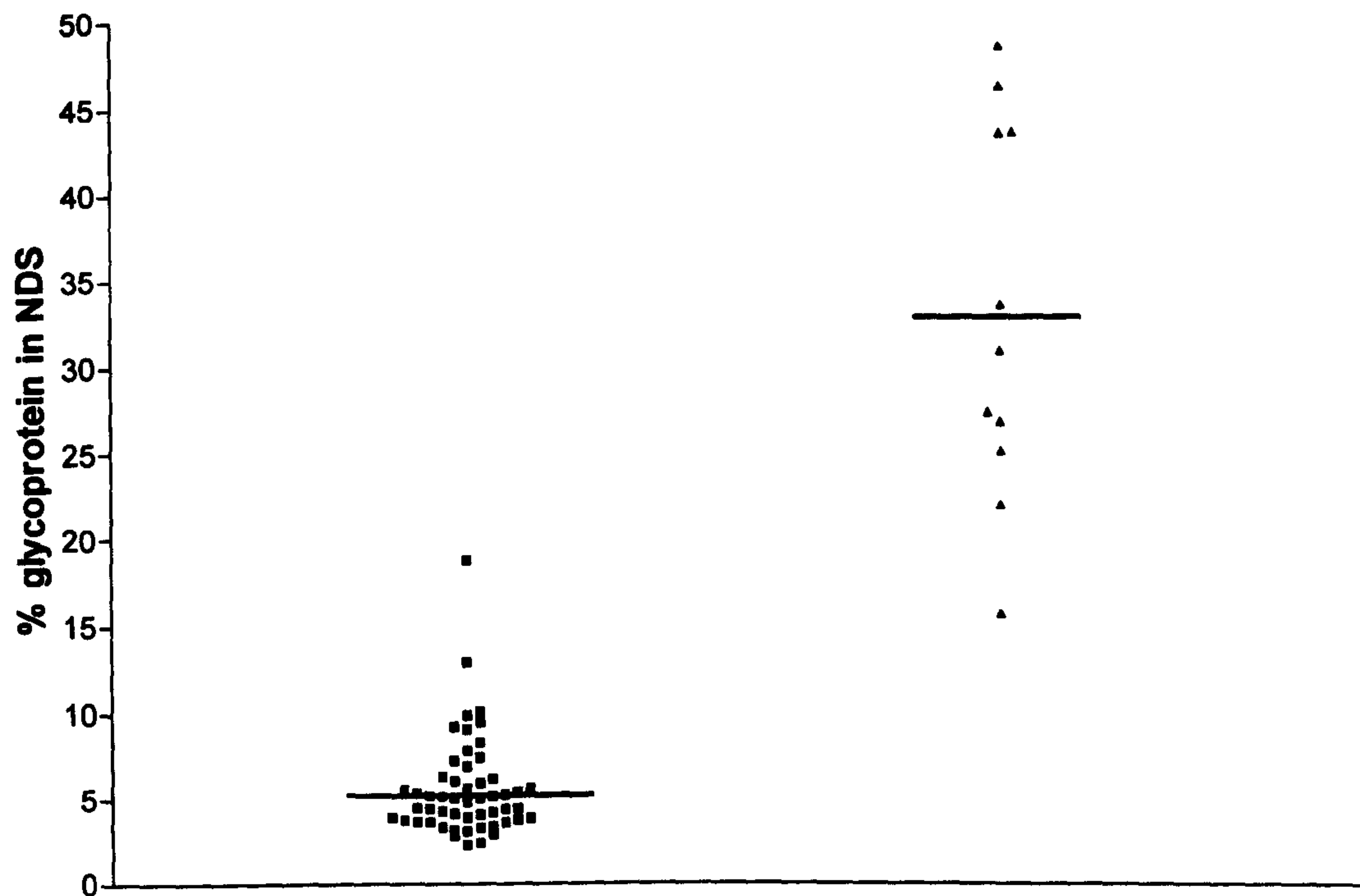
15.54 to 48.55% of NDS with a mean of 32.97 ± 3.31 (n=11) (Figure 5.3). There was a significant difference in glycoprotein content of NDS between the two groups ($P < 0.0001$, unpaired t test). The addition of proteolytic inhibitors during isolation gives a 5.9 fold underestimation of the glycoprotein content, consequently for all further experiments using effusion mucin isolated in proteolytic inhibitor buffer, the values derived from the PAS assay have been corrected by this factor.

Figure 5.2 Non-dialysable solids (NDS) content (mg) of effusions



Effusions homogenised in proteolytic inhibitor buffer (■) n=54, or distilled water (▲) n=11, dialysed, freeze dried and weighed. There was no significant difference in NDS content between the two groups (P=0.628, unpaired t test).

Figure 5.3 Glycoprotein content of effusion non-dialysable solids (NDS)



A known amount of each effusion NDS, previously homogenised in proteolytic inhibitor buffer (■) n=54 or in distilled water (▲) n=11, was resolubilised and analysed for the presence of glycoprotein using the PAS method (Mantle and Allen, 1978). There was a significant difference in glycoprotein content of NDS between the two groups ($P<0.0001$, unpaired t test) leading to an approximate 5.9 fold underestimation in glycoprotein content in those samples homogenised in proteolytic inhibitor buffer.

5.2.3 Rheological properties and glycoprotein composition of effusions

5.2.3.1 *Effusion rheology*

In the following study a reduced number of effusions (n=50) were used because all parameters were not measured in all samples. Thirty-six of these effusions were classified by clinical inspection as mucoid (thick), the remainder were serous (thin).

The specific viscosities of homogenised effusions (n=50) were measured at pH6.5 and 37°C in the presence of proteolytic inhibitors using a Contraves low shear viscometer (section 2.5.1). The viscosities of the homogenates varied between 0.06 and 0.22 with a mean of 0.11 ± 0.017 (n=11) for serous effusions and between 0.12 and 5.0 with a mean of 1.24 ± 0.21 (n=39) for mucoid effusions. There was a significant difference ($P=0.0061$, unpaired t test) between the viscosities of the two types of effusion.

In order to ascertain which of the components present in the effusions influence the viscosity, the content of each component was plotted against specific viscosity and the statistical correlation was determined.

5.2.3.2 *Effusion non-dialysable solids*

The content of NDS in the effusions was determined as before by weighing the lyophilised homogenates after exhaustive dialysis against distilled water at 4°C and freeze-drying. The NDS ranged from 0.49 to 41.3mg with a mean of 13.29 ± 1.27 (n=50).

There was a significant correlation between the specific viscosity of each effusion homogenate and the NDS content ($P=0.0002$, Pearson correlation) (Figure 5.4). This result demonstrates a dilution effect, as viscosity depends on a number of parameters

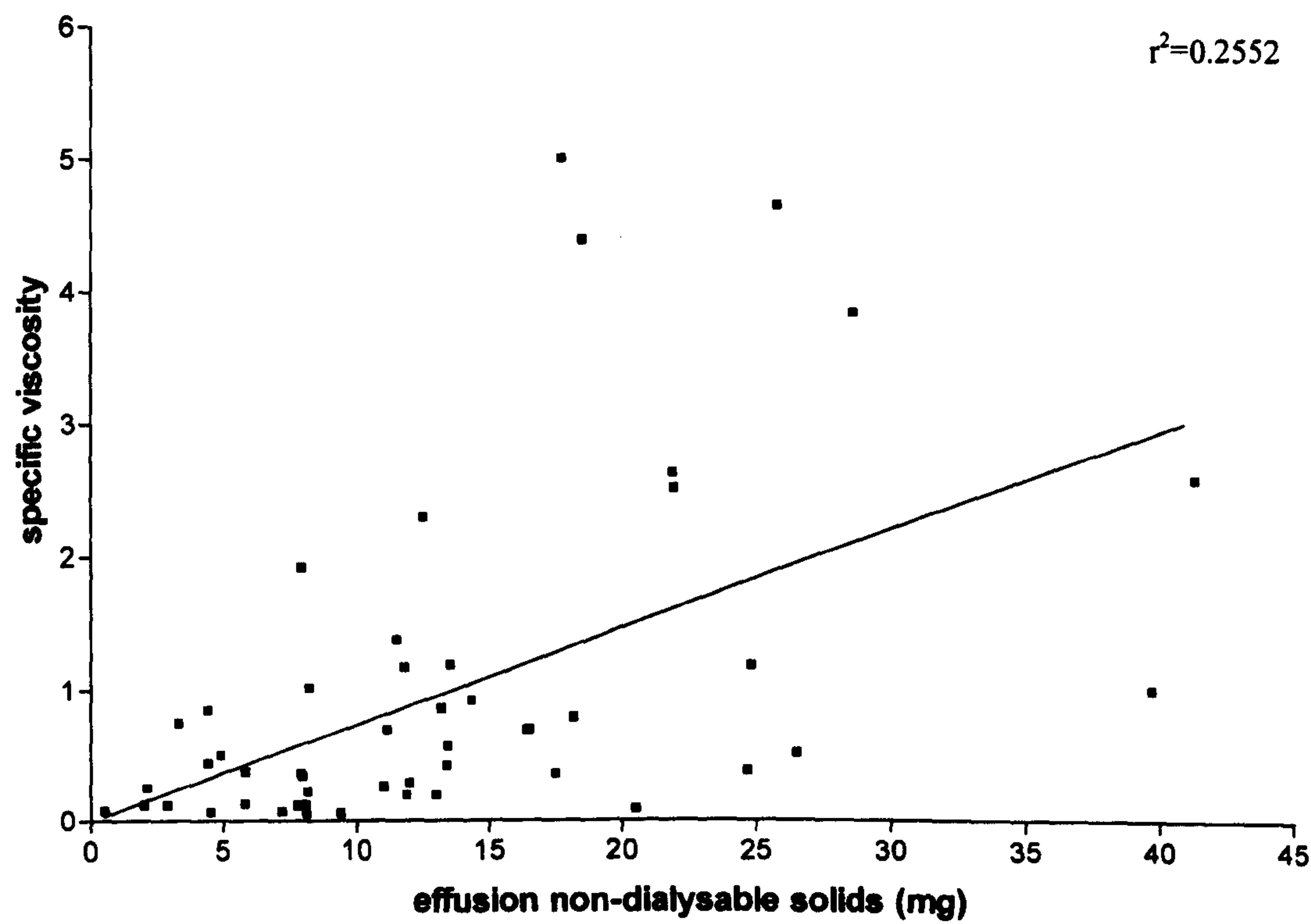
including the concentration of the solution, in general the more concentrated the solution the more viscous it is.

5.2.3.3 *Effusion glycoprotein content*

The glycoprotein content of the effusion homogenates was determined by the modified PAS method (Mantle and Allen, 1978), after resolubilising a known amount of each effusion NDS in distilled water. The glycoprotein content was expressed in mg per effusion and ranged from 0.31 to 13.05mg with a mean of 3.84 ± 0.44 (n=50).

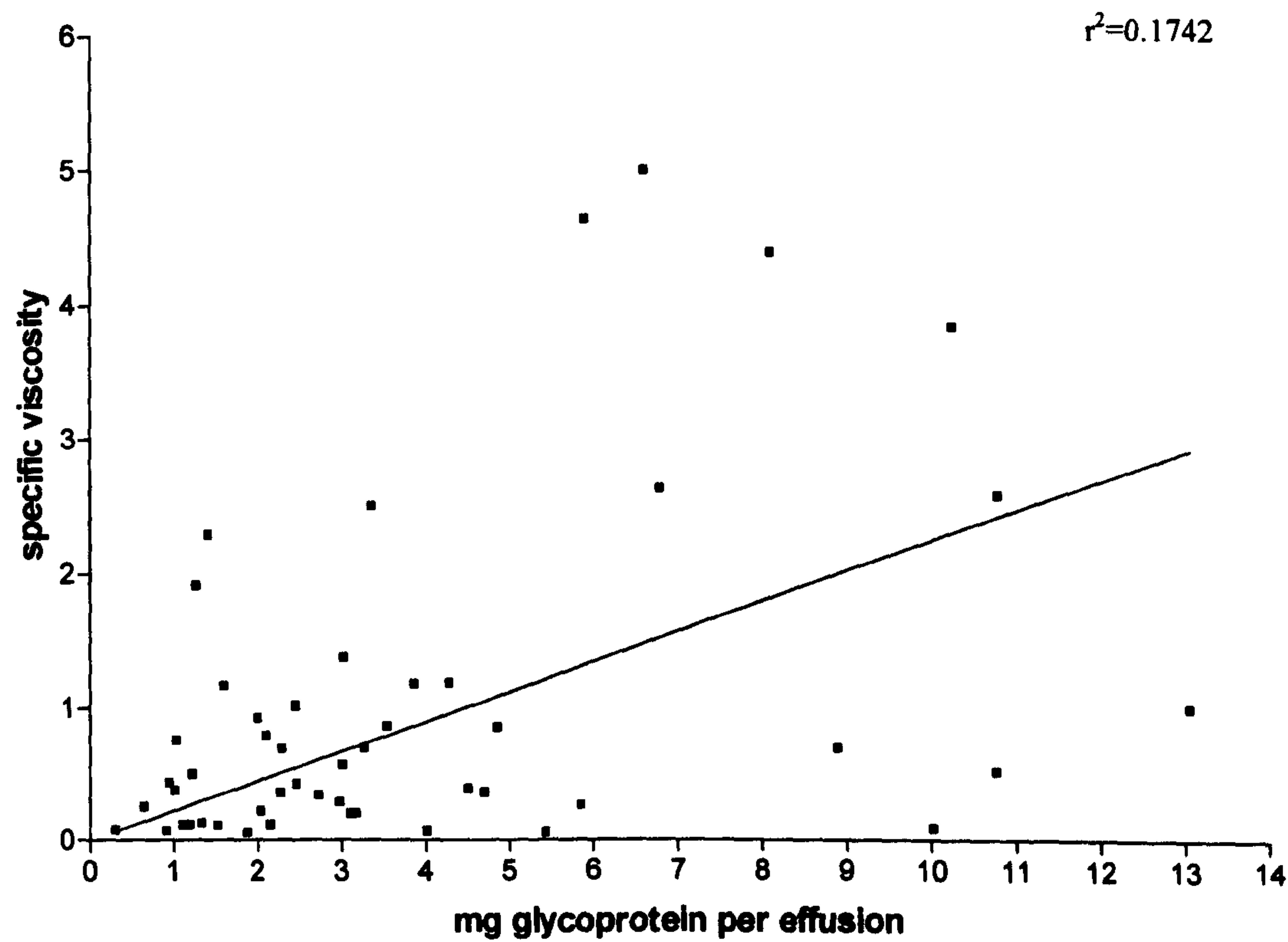
There was a significant correlation between the specific viscosity of each effusion homogenate and the glycoprotein content of the effusion ($P=0.0026$, Pearson correlation) (Figure 5.5). This can easily be explained, as glycoprotein is a polymer and in high glycoprotein concentration solutions, such as in middle ear effusions, interactions between the polymer chains will increase the viscosity of the solution.

Figure 5.4 The relationship between specific viscosity at pH6.5, 37°C of middle ear effusion homogenates and NDS content



Non-dialysable solids content (mg) per effusion (■) was plotted against specific viscosity. There was a significant correlation between the two parameters (P=0.0002, Pearson correlation).

Figure 5.5 The relationship between specific viscosity at pH6.5, 37°C of middle ear effusion homogenates and glycoprotein content



Glycoprotein content (mg) per effusion (■) was plotted against specific viscosity. There was a significant correlation between the two parameters ($P=0.0026$, Pearson correlation).

5.2.3.4 Purification and analysis of mucin from effusions

For the analysis of mucin from effusions the thick effusion samples only (n=36) were used as they contain more mucin. It has been previously demonstrated that thin effusions have only 57% of the mucin of thick effusions (Fitzgerald *et al.*, 1988).

The remaining lyophilised material from each thick effusion was solubilised in 0.2M NaCl/0.02% NaN₃ and caesium chloride was added to give a starting density of 1.42gml⁻¹. The effusions were centrifuged at 100,000g for 48h at 4°C using a fixed angle rotor (section 2.5.3). This CsCl purification procedure gives clear separation of glycoprotein material from protein contamination. The content of each centrifuge tube was separated into nine equal fractions, the density of each fraction was measured and those with densities of 1.42–1.55gml⁻¹ (the characteristic buoyant density range for glycoprotein (Fitzgerald *et al.*, 1987)) were pooled for each effusion. After exhaustive dialysis at 4°C against distilled water and freeze-drying, the glycoprotein present in each effusion was blotted onto nitrocellulose sheets (0.45µm) and incubated overnight at 4°C in PBS containing 2% (w/v) BSA. The glycoprotein was then probed with antibodies raised to the human MUC5AC (NCL-HGM-45M1) and MUC5B gene products (TEPA) (section 2.4.2.1) in a slot blot ELISA assay along with the appropriate CsCl purified standards (pig gastric mucin for MUC5AC and human salivary mucin for MUC5B (Chapter 4)). Antibody binding was measured with the relevant secondary antibodies conjugated to peroxidase with H₂O₂ as substrate and 3,3'-diaminobenzidine hydrochloride for colour development. Staining of the slots was quantitated using a Shimadzu scanning densitometer at 595nm.

From the ELISAs, the amount of MUC5B and MUC5AC present in 100µg of each CsCl purified effusion was determined, expressed as a percentage, and then the content (mg) of each mucin per effusion was calculated from the total glycoprotein content (Table 5.2). Two of the 36 effusions were excluded from the data as the levels of MUC5B fell out of the normal range and were 6.5 and 20.6-fold higher than the others.

MUC5B was present in all 34 effusions in the range 0.006 to 11.35mg with a mean of 2.13 ± 0.46 (n=34).

MUC5AC was present in thirty-two of thirty-four effusions in the range 0.002 to 1.56mg with a mean of 0.16 ± 0.06 (n=32). MUC5AC was present in much smaller amounts than MUC5B.

The mucin content of each effusion was plotted against specific viscosity and the statistical correlation determined. There was a significant correlation between MUC5B and specific viscosity ($P < 0.0001$, Pearson correlation) (Figure 5.6) but no correlation existed between MUC5AC and specific viscosity ($P = 0.503$, Pearson correlation) (Figure 5.7).

The above data demonstrates that effusion viscosity is related to the amount of MUC5B present (without taking into account the concentration). Effusion volumes varied between 0.042 and 0.51ml and were solubilised in 2ml proteolytic inhibitor buffer therefore volume differences would have only a small effect on mucin concentrations.

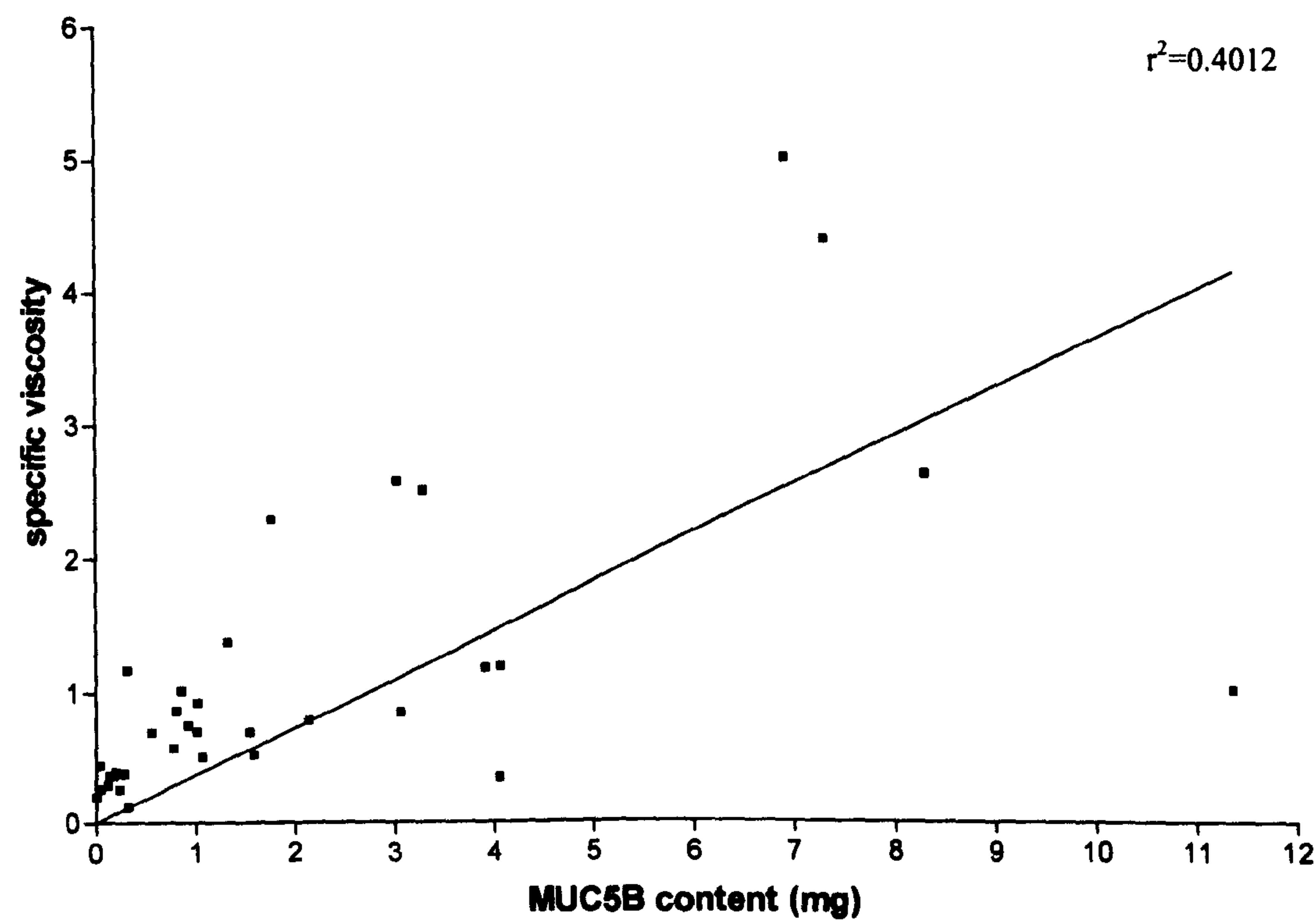
The amount of glycoprotein present in the effusions is important and the above data can also be expressed using the mucin concentration of the homogenates as they were measured. The mucin concentration of each effusion was calculated (mg MUC5B or MUC5AC per effusion / effusion homogenate volume) and plotted against specific

viscosity. The statistical correlation was determined and the results demonstrated that there was a significant correlation between MUC5B concentration and specific viscosity ($P=0.0009$, Pearson correlation) (Figure 5.8) but no correlation existed between MUC5AC concentration and specific viscosity ($P=0.7155$, Pearson correlation) (Figure 5.9).

Table 5.2 MUC5B and MUC5AC content of effusions

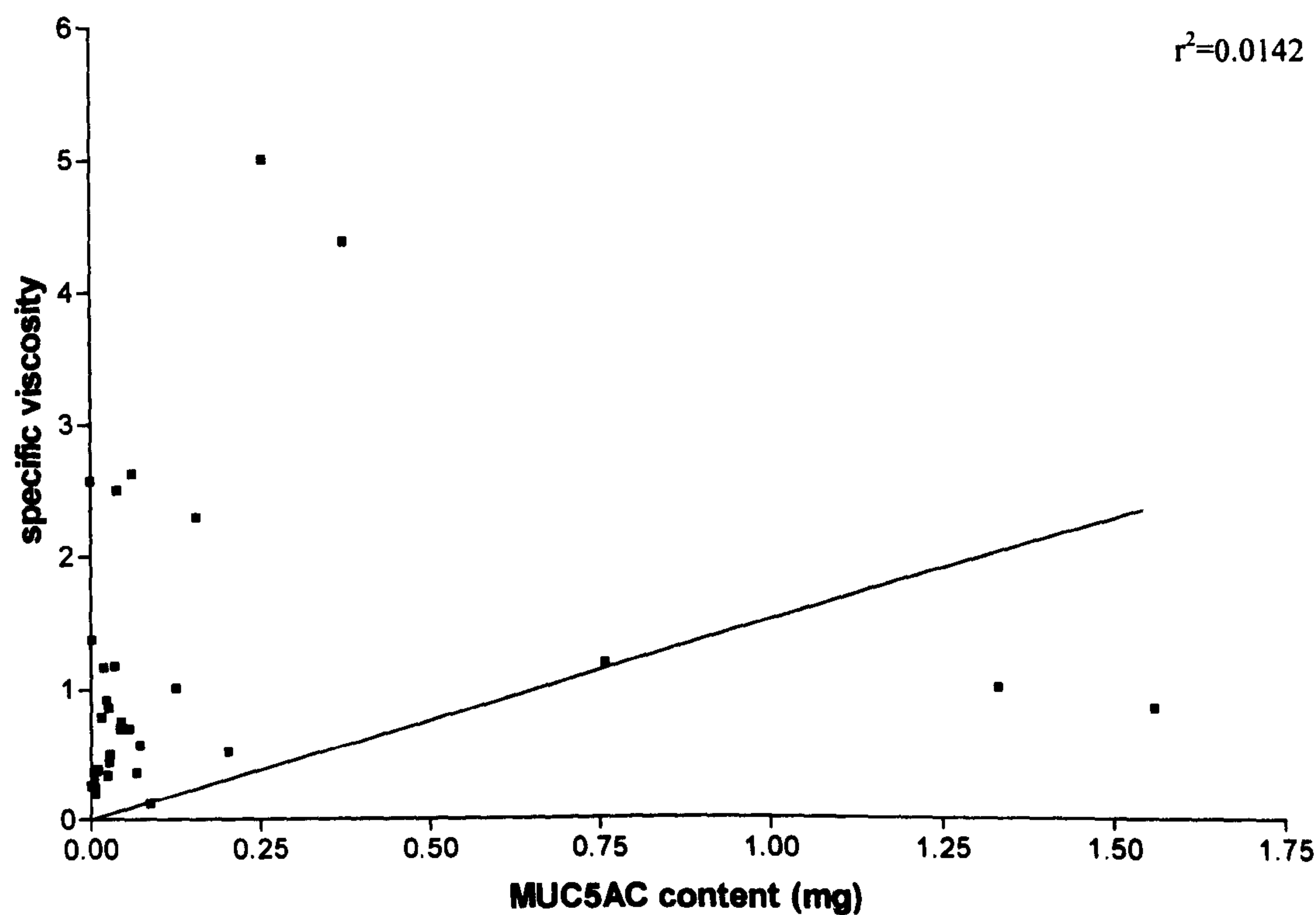
Mucin (µg) in 100µg CsCl purified mucin		Mucin (mg) per effusion	
MUC5B	MUC5AC	MUC5B	MUC5AC
90	4.4	0.929	0.045
4.5	2.9	0.042	0.027
35	5.1	0.853	0.126
44	0.05	1.33	0.0016
38	1.1	0.24	0.0073
26	2.4	0.78	0.0731
88	2.4	1.072	0.0287
31	1.4	1.014	0.0437
5.8	2.9	0.13	0.0676
90.2	4.6	7.30	0.372
104.7	3.8	6.91	0.254
0.18	0.2	0.0056	0.007
27.4	7.4	0.32	0.087
17.4	0.6	1.54	0.0566
14.7	1.9	1.59	0.203
122.2	0.9	8.29	0.061
0.8	0	0.047	0
28	0	3.02	0
63	32.1	3.06	1.56
87	10.2	11.35	1.33
95	17.7	4.06	0.757
22.8	0.75	0.81	0.264
101.2	0.9	3.91	0.357
3.9	0.083	0.18	0.0038
148.6	0.92	4.05	0.025
125.5	11.1	1.76	0.156
4.6	0.24	0.21	0.011
24.5	2.03	0.56	0.046
98	1.2	3.29	0.040
102.3	0.75	2.14	0.0157
28.4	0.82	0.29	0.008
51	1.2	1.02	0.023
20	1.24	0.32	0.0198
4.3	0.18	0.13	0.0052

Figure 5.6 The relationship between specific viscosity at pH6.5, 37°C of middle ear effusion homogenates and MUC5B content



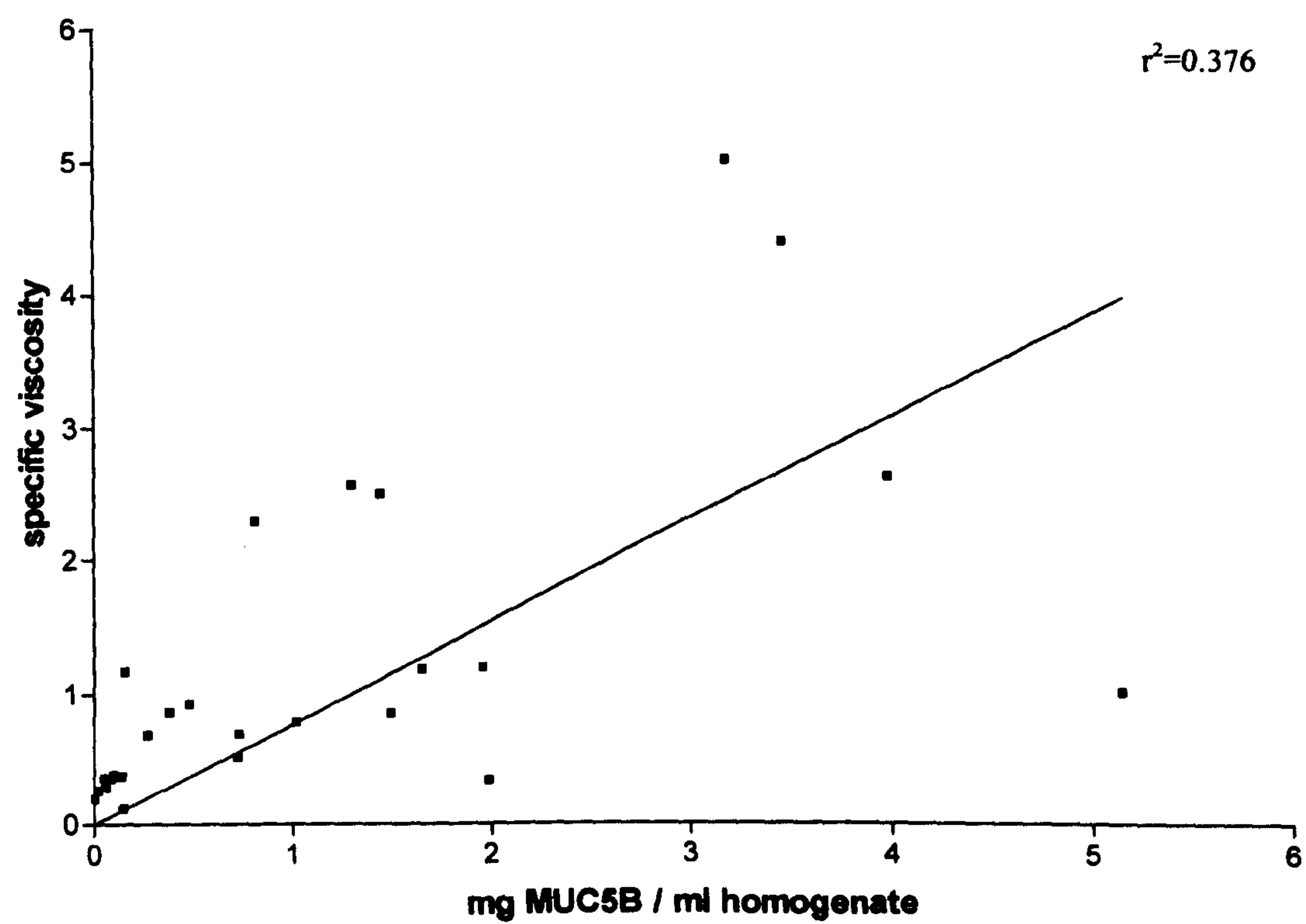
CsCl purified mucins were blotted onto nitrocellulose membranes and tested for MUC5B by reactivity with TEPA in a slot blot ELISA. Staining of the slots was quantitated and the amount of MUC5B present in each effusion was calculated and plotted against specific viscosity. A significant correlation was observed between MUC5B and specific viscosity ($P<0.0001$, Pearson correlation).

Figure 5.7 The relationship between specific viscosity at pH6.5, 37°C of middle ear effusion homogenates and MUC5AC content



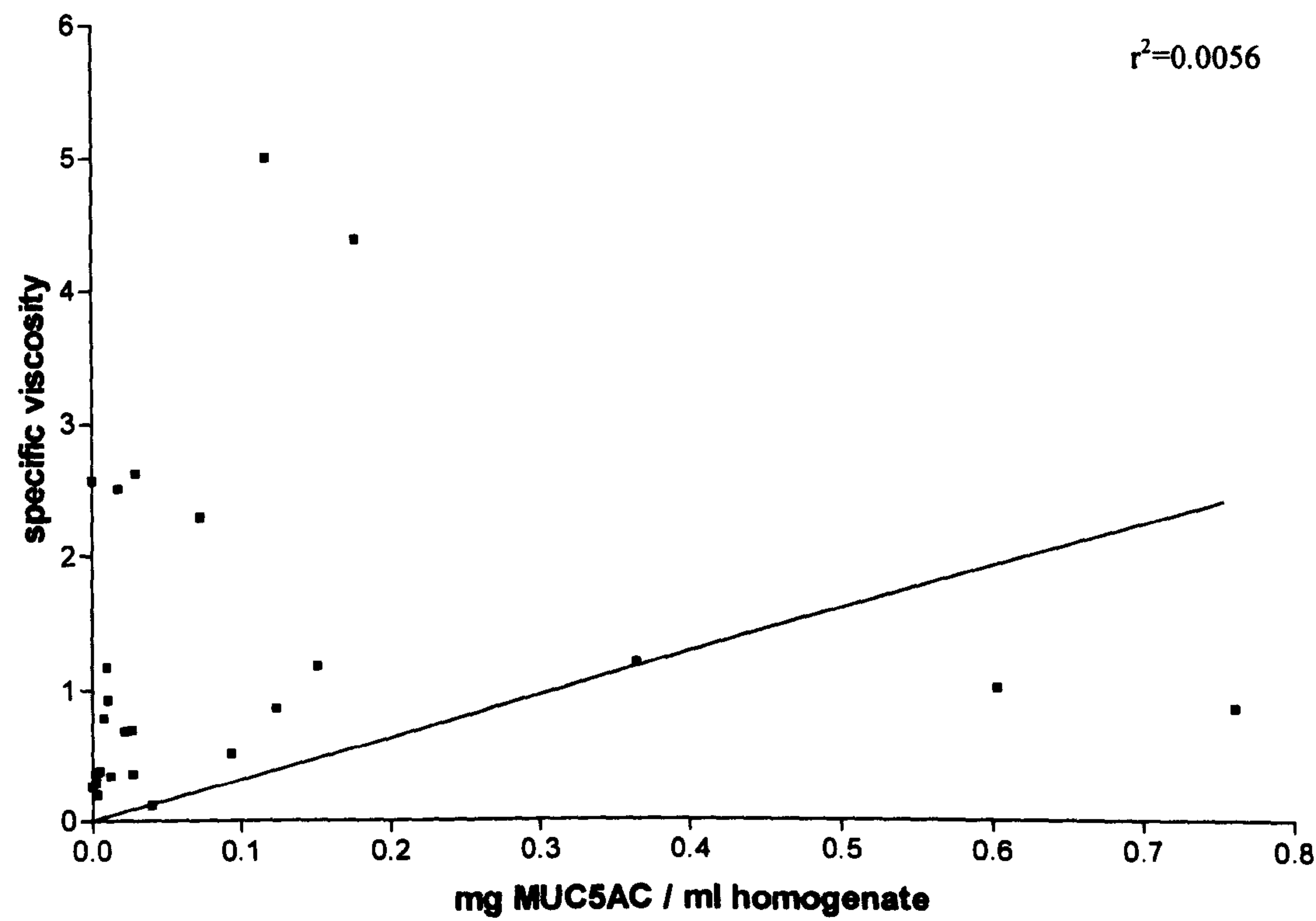
CsCl purified mucins were blotted onto nitrocellulose membranes and tested for MUC5AC by reactivity with NCL-HGM-45M1 in a slot blot ELISA. Staining of the slots was quantitated and the amount of MUC5AC present in each effusion was calculated and plotted against specific viscosity. There was no significant correlation between MUC5AC and specific viscosity ($P=0.503$, Pearson correlation).

Figure 5.8 The relationship between specific viscosity at pH6.5, 37°C of middle ear effusion homogenates and MUC5B concentration



The MUC5B concentration of each effusion homogenate was calculated using the effusion homogenate volume and plotted against specific viscosity. A significant correlation was observed between MUC5B concentration and specific viscosity (P=0.0009, Pearson correlation).

Figure 5.9 The relationship between specific viscosity at pH6.5, 37°C of middle ear effusion homogenates and MUC5AC concentration



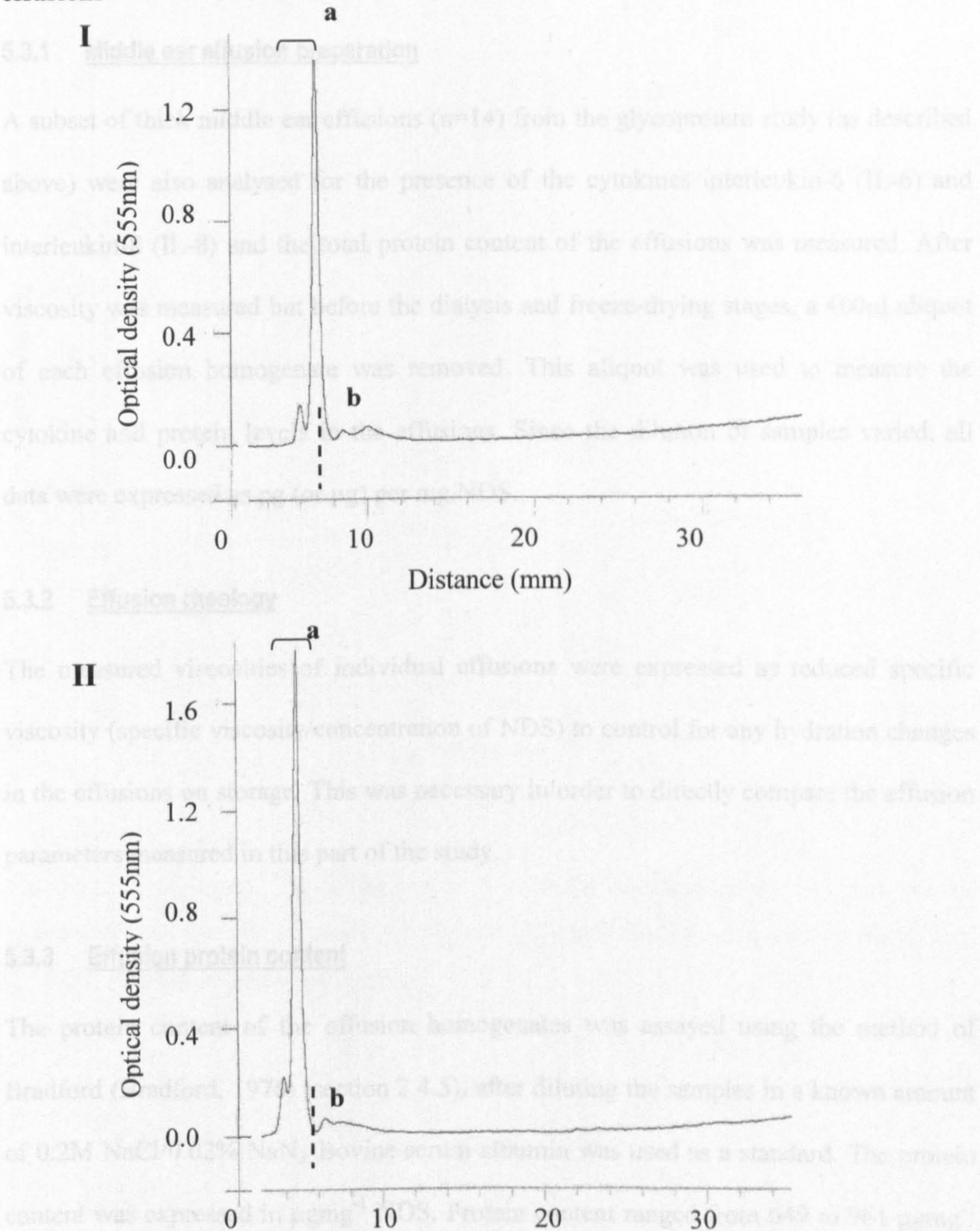
The MUC5AC concentration of each effusion homogenate was calculated using the effusion homogenate volume and plotted against specific viscosity. No significant correlation was observed between MUC5AC concentration and specific viscosity (P=0.7155, Pearson correlation).

5.2.4 SDS PAGE of mucin from effusions

SDS PAGE was used to confirm the size and degradation state of the mucin from thick effusions. Effusion mucin purified by CsCl density centrifugation that remained after ELISA (section 5.2.3.4) was used. Samples (1 μ l) of effusion mucin (10mgml⁻¹ non-reducing buffer) were subjected to gel electrophoresis using a 4-15% gradient gel. Gels were stained for glycoprotein using the PAS method (Van Seuningen and Davril, 1992) and scanned at 555nm.

Following gel electrophoresis, purified effusion mucin largely remained ($83.5 \pm 2.4\%$) within the stacking gel as two peaks around the point of application (Figure 5.10). All peaks within the stacking gel were denoted as band “a”. A small proportion of mucin (approximately 16%), clearly separate from band “a” and representing lower molecular size material, peaked in the running gel adjacent to the interface of the stacking and running gel. This material was denoted band “b”. There was no other PAS staining material present further into the running gel than band “b”, which showed the purified mucins to be free of any smaller molecular weight glycoproteins. All effusions tested gave a similar pattern.

Figure 5.10 SDS PAGE of non-reduced CsCl purified effusion mucin from thick effusions



Purified mucin, (after CsCl centrifugation) in non-reducing buffer from two different thick effusions. Band “a”, mucin within stacking gel, band “b”, mucin spreading into the running gel from the interface. The dashed line shows the position of the interface between the stacking and running gel.

5.3 Analysis of cytokines present in middle ear effusions

5.3.1 Middle ear effusion preparation

A subset of thick middle ear effusions (n=14) from the glycoprotein study (as described above) were also analysed for the presence of the cytokines interleukin-6 (IL-6) and interleukin-8 (IL-8) and the total protein content of the effusions was measured. After viscosity was measured but before the dialysis and freeze-drying stages, a 400µl aliquot of each effusion homogenate was removed. This aliquot was used to measure the cytokine and protein levels in the effusions. Since the dilution of samples varied, all data were expressed as pg (or µg) per mg NDS.

5.3.2 Effusion rheology

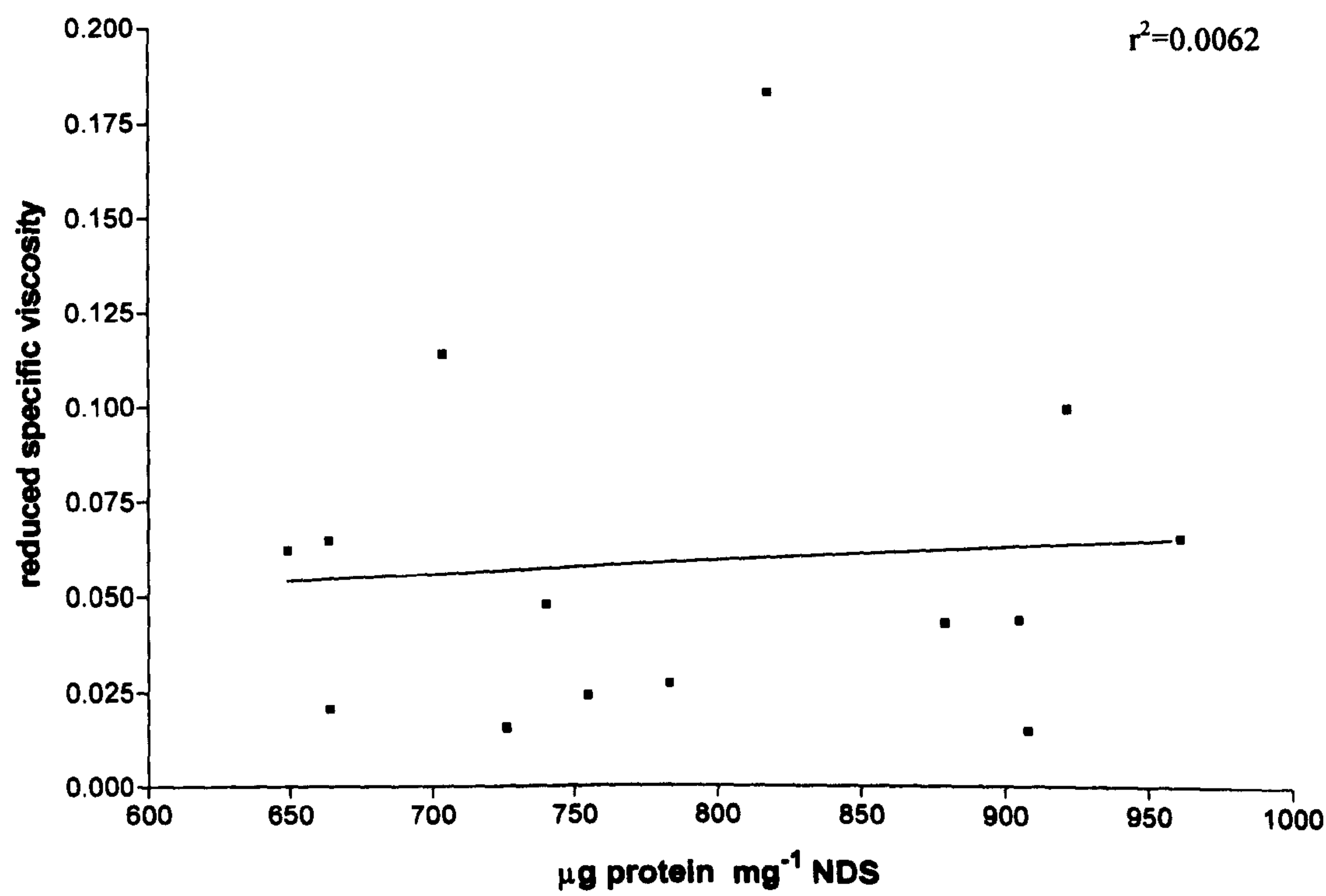
The measured viscosities of individual effusions were expressed as reduced specific viscosity (specific viscosity/concentration of NDS) to control for any hydration changes in the effusions on storage. This was necessary in order to directly compare the effusion parameters measured in this part of the study.

5.3.3 Effusion protein content

The protein content of the effusion homogenates was assayed using the method of Bradford (Bradford, 1976) (section 2.4.5), after diluting the samples in a known amount of 0.2M NaCl/0.02% NaN₃. Bovine serum albumin was used as a standard. The protein content was expressed in µgmg⁻¹ NDS. Protein content ranged from 649 to 961 µgmg⁻¹ NDS with a mean of 791 ± 29 (n=14).

There was no correlation between the reduced specific viscosity and the protein content of each effusion homogenate (P=0.79, Pearson correlation) (Figure 5.11).

Figure 5.11 The relationship between reduced specific viscosity of effusion homogenates and protein content



Protein content $\mu\text{gmg}^{-1}\text{ NDS}$ (■) for each effusion homogenate (n=14) was plotted against reduced specific viscosity. There was no correlation between the two parameters (P=0.79, Pearson correlation)

5.3.4 Effusion cytokine content

The IL-6 and IL-8 content of effusions were measured by probing known volumes of the effusion homogenates and relevant standards with antibodies raised to the human IL-6 and IL-8 gene products using a quantitative sandwich enzyme immunoassay technique (section 2.4.3). Each cytokine was expressed as pgmg^{-1} NDS.

IL-6 ranged from 1.5 to 558.5pgmg^{-1} NDS with a mean of 178 ± 42.5 (n=14). IL-8 ranged from 61.8 to 17130pgmg^{-1} NDS with a mean of 4020 ± 1317 (n=14). The content of the cytokines in each effusion were plotted against each other and against reduced specific viscosity to determine if any correlation existed.

There was a significant correlation between the IL-6 and IL-8 content in effusions ($P=0.0149$, Pearson correlation) (Figure 5.12). This result demonstrates that one of these cytokines may be regulated by the other or that they are both regulated by a common factor such as another cytokine. There was no correlation between IL-6 and reduced specific viscosity (Figure 5.13), however a significant correlation did exist between IL-8 and reduced specific viscosity ($P=0.0267$, Pearson correlation) (Figure 5.14) if one point was excluded.

Previous studies have expressed cytokine levels in effusions per mg protein and the results were also presented in this way to enable a direct comparison between this and previous results.

Effusion	IL-6 per mg protein	IL-8 per mg protein
1	47.32	813.99
2	120.41	930.10
3	224.38	1456.661
4	2.27	93.05
5	140.89	1518.05
6	195.42	12340.02
7	254.48	1604.40
8	90.64	13922.66
9	513.92	5323.46
10	124.84	1176.32
11	246.12	7640.05
12	30.33	522.29
13	542.54	5594.24
14	615.10	18869.25

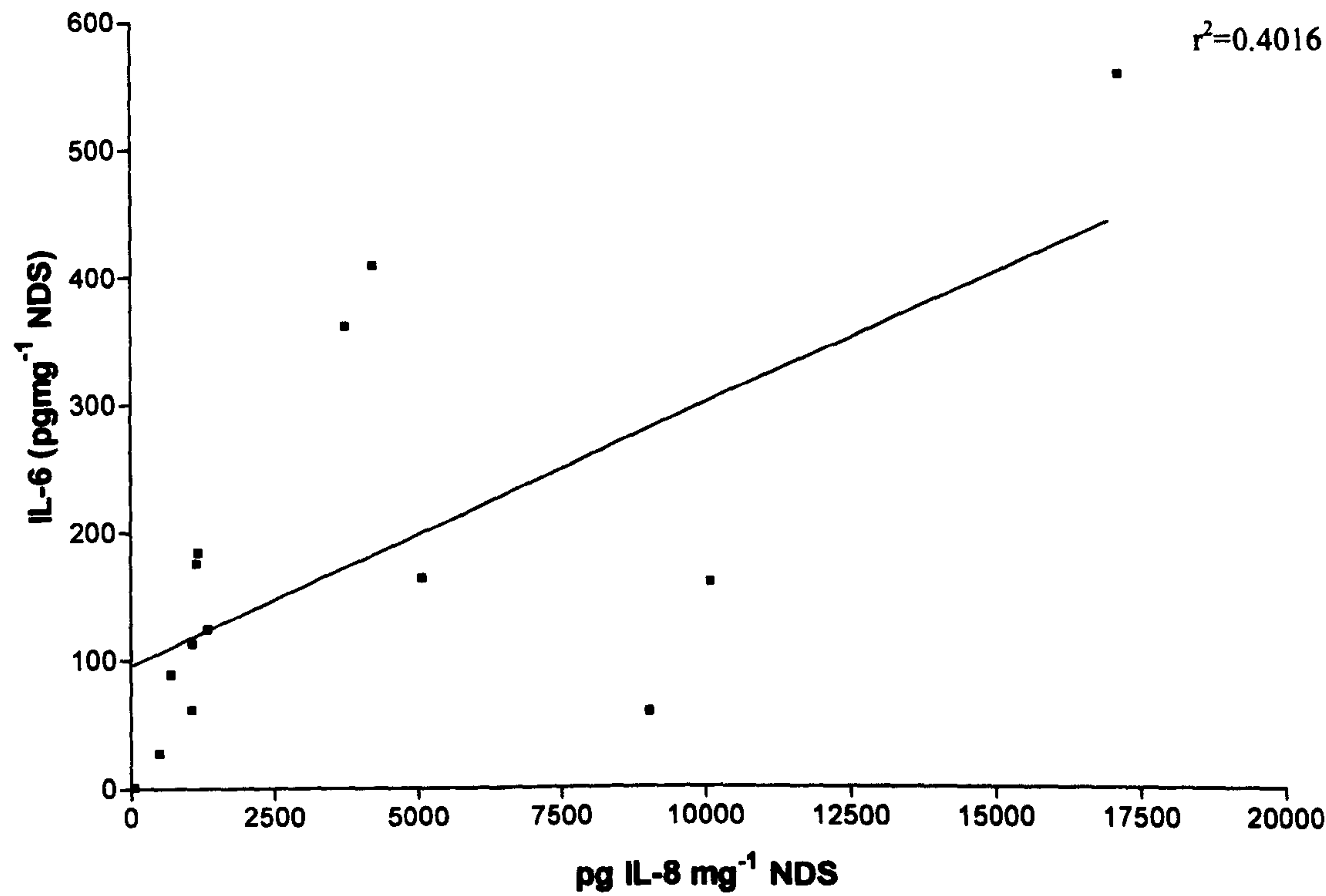
IL-6 ranged from 2.27 – 615.1pgmg⁻¹ protein with a mean of 224.9 ± 52.62 (n=14).

IL-8 ranged from 93.05 to 18 870pgmg⁻¹ protein with a mean of 5129 ± 1593 (n=14).

The IL-6 content of effusions was generally much lower than IL-8 content, irrespective of the method of expressing the data.

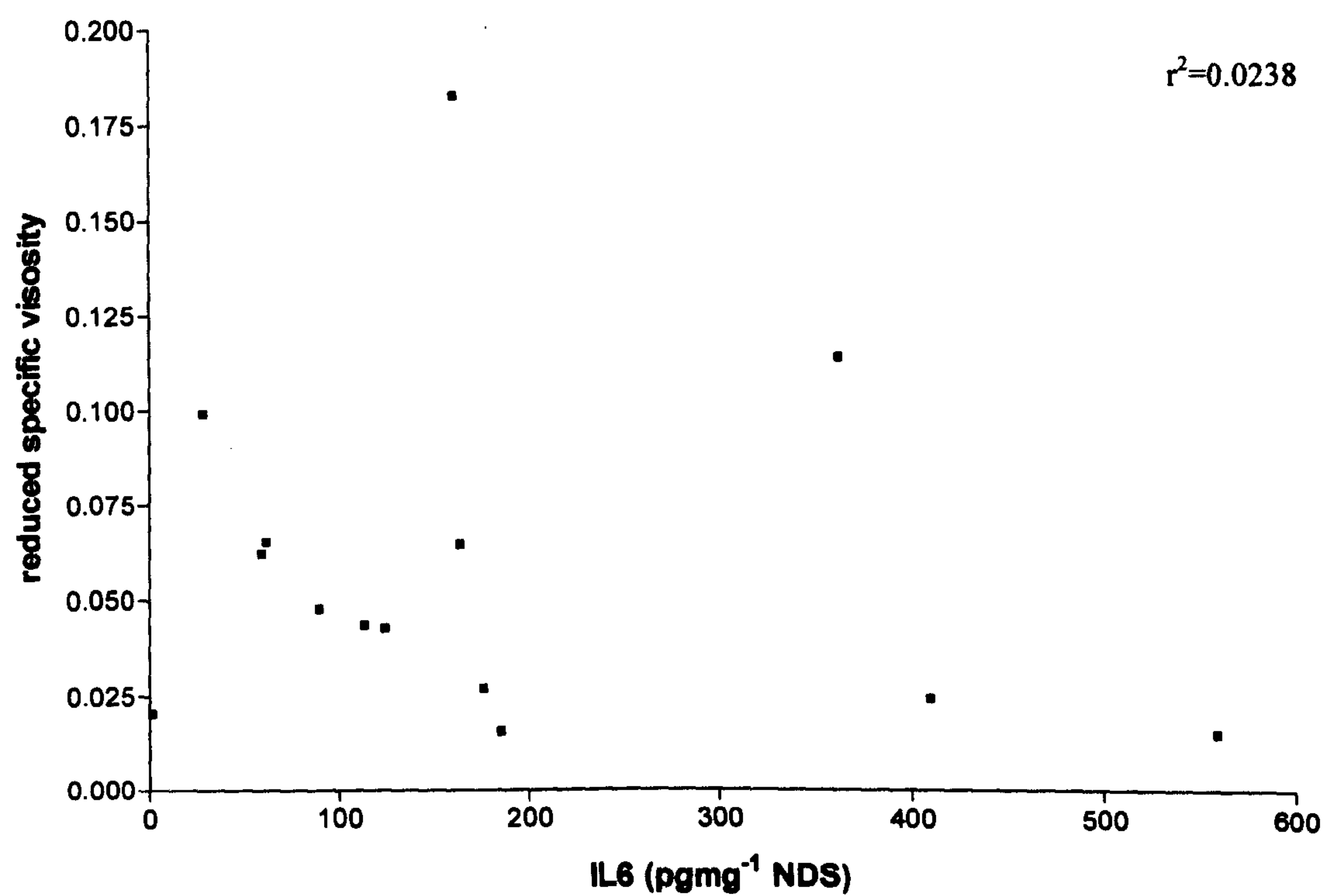
Previous studies have reported IL-6 levels in effusions of $173.9 \pm 75 \text{pgmg}^{-1}$ protein (Yellon *et al.*, 1992) and IL-8 levels of $4805 \pm 913 \text{pgmg}^{-1}$ protein and $575 \pm 50 \text{pgmg}^{-1}$ protein (Maxwell *et al.*, 1994; Johnson *et al.*, 1997) and the results shown in this current study fit very well with the above.

Figure 5.12 Correlation of IL-6 and IL-8 content in effusions



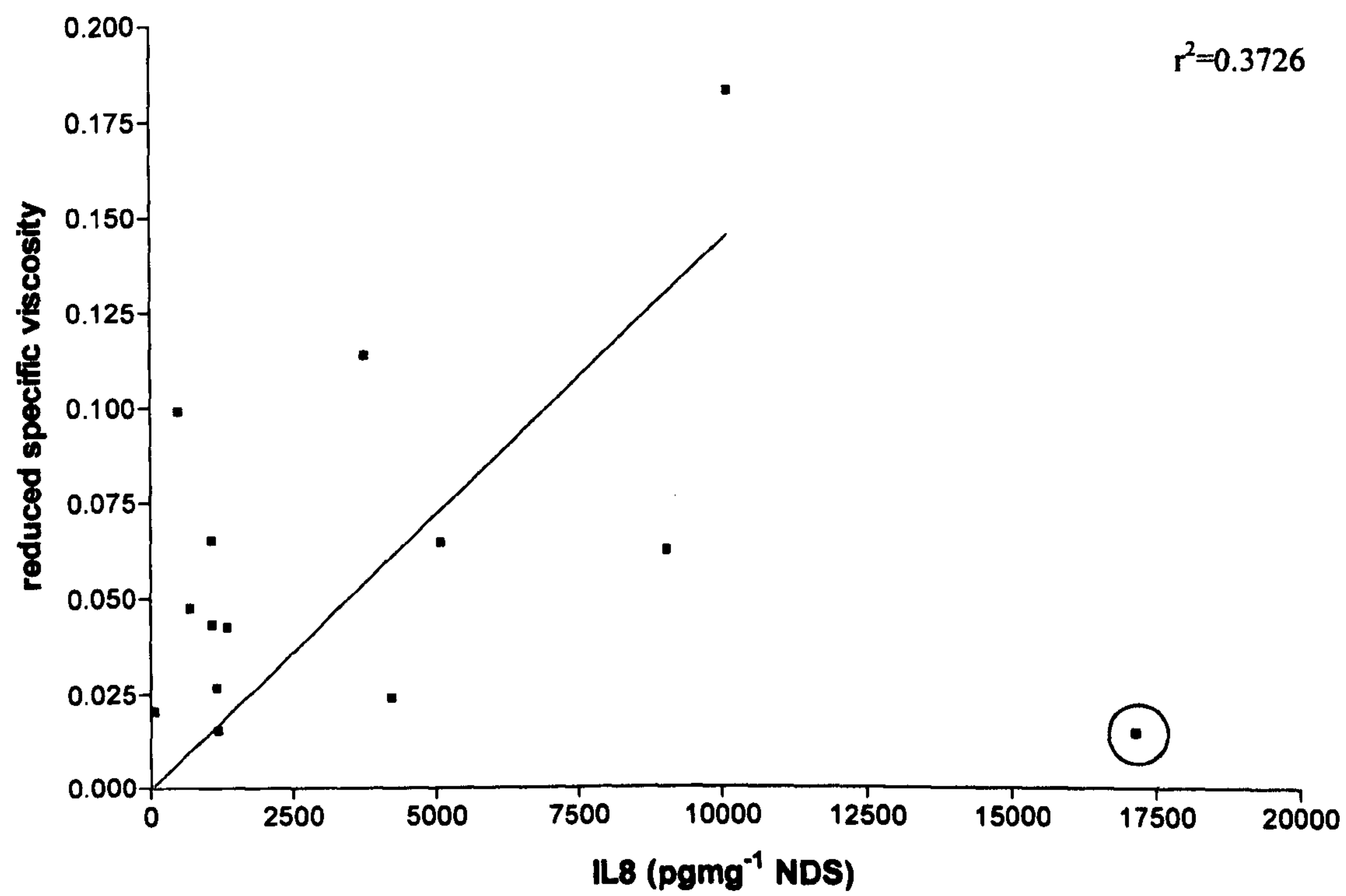
IL-6 content of effusions (pgmg⁻¹ NDS) was plotted against IL-8 content (pgmg⁻¹ NDS) (■) and the statistical correlation was determined. There was a significant correlation between the levels of the two cytokines (P=0.0149, Pearson correlation).

Figure 5.13 The relationship between reduced specific viscosity at pH6.5, 37°C of middle ear effusion homogenates and IL-6 content



IL-6 content of effusions (pgmg⁻¹ NDS) was plotted against reduced specific viscosity (■) and the statistical correlation was determined. There was no correlation between the IL-6 content and reduced specific viscosity in effusions (P=0.599, Pearson correlation).

Figure 5.14 The relationship between specific viscosity at pH6.5, 37°C of middle ear effusion homogenates and IL-8 content



IL-8 content of effusions (pgmg⁻¹ NDS) was plotted against reduced specific viscosity (■) and the statistical correlation was determined. There was a significant correlation between the IL-8 content and reduced specific viscosity in effusions (P=0.0267, Pearson correlation), but only when one point (circled) was excluded.

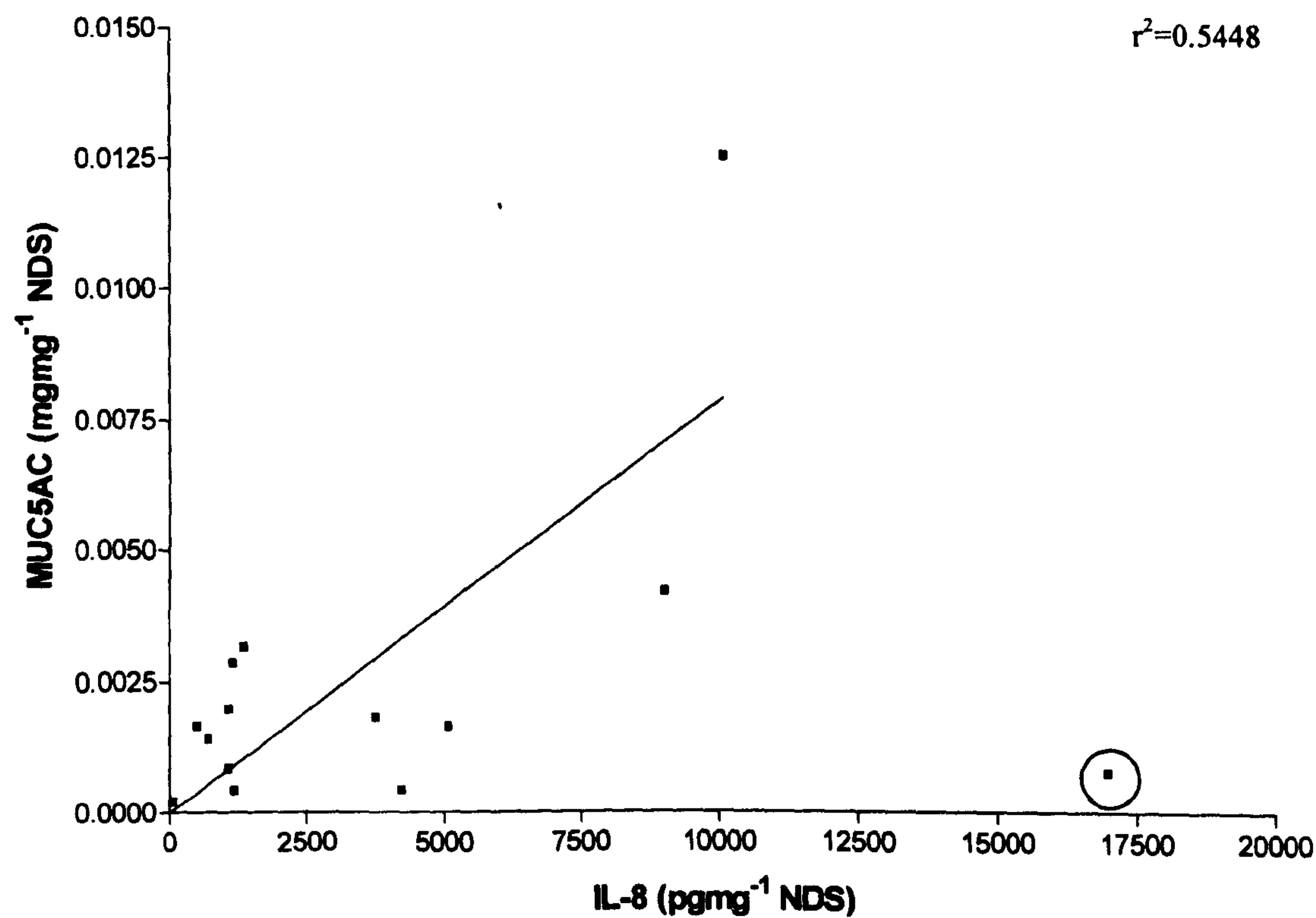
5.3.5 Is there a relationship between cytokine levels and the amount and type of mucin present in effusions?

The MUC5B and MUC5AC content of each effusion (previously measured in section 5.2.3.3 but calculated as mgmg^{-1} NDS for this part of the study) were plotted against both the IL-6 and IL-8 content and the statistical correlations were determined.

Parameters	P value	Is correlation significant?
MUC5B and IL-6	0.6169	No
MUC5B and IL-8	0.5085	No
MUC5AC and IL-6	0.6220	No
MUC5AC and IL-8	0.004	Yes

MUC5AC had a significant correlation against IL-8 content in effusions (Figure 5.15), but only when one point was excluded, (which was the same point as excluded in Figure 5.14). There were no other significant correlations between the parameters.

Figure 5.15 Correlation of MUC5AC and IL-8 content of effusion homogenates



IL-8 content of effusions (pgmg⁻¹ NDS) was plotted against MUC5B content (mgmg⁻¹ NDS) (■) and the statistical correlation was determined. There was a significant correlation between the IL-8 content and MUC5B in effusions (P=0.004, Pearson correlation), but only when one point (circled) was excluded.

5.4 Discussion

In this study, the rheological and biochemical properties of individual middle ear effusions were determined.

Middle ear effusions are generally very small and viscous therefore for accurate viscosity measurements each sample was diluted in 2ml 0.067M phosphate buffer. The buffer contained a wide range of proteolytic inhibitors (Fitzgerald *et al.*, 1987) to minimise *in vitro* degradation by endogenous enzymes, which would affect the rheological properties of the samples. This extraction buffer has been shown to inhibit *in vitro* proteolysis during the isolation of cartilage proteoglycans (Pearson and Mason, 1977), which are high molecular weight glycoconjugates, as are mucin glycoproteins. Mild homogenisation of the effusion has been shown to be necessary to solubilise the entire mucin population (Fitzgerald *et al.*, 1987).

Samples were collected from two different centres (Freeman Hospital, Newcastle and Queens Medical Centre, Nottingham) and it was impossible to measure the effusion viscosity immediately. The samples were stored frozen at -20°C until used, conditions that would inevitably lead to some dehydration. This effect has been taken into account by the expression of biochemical analyses (cytokines and protein only) using non-dialysable solids.

In the preliminary study of the effect of proteolytic inhibitors it was found that middle ear effusions (n=54) isolated in the presence of a cocktail of inhibitors (and after dialysis and freeze drying) had a significantly lower level of measurable glycoprotein using the PAS assay than effusions isolated in distilled water (n=11), however the content of NDS in the two groups were similar (11.98mg and 14.18mg, respectively).

There was a 5.9 fold reduction in the glycoprotein estimation in effusions isolated in the presence of inhibitors compared to those isolated in distilled water. Detection of a known amount of papain digested SIGMA pig gastric mucin alone in the same assay system was similar regardless of whether inhibitors were present or not and was therefore unaffected by treatment using inhibitors. Previous studies have demonstrated that up to 32% of the NDS of effusions is glycoprotein (Fitzgerald *et al.*, 1989; Carrie *et al.*, 1992). The presence of the other effusion components is probably necessary to cause the proteolytic inhibitor interactions.

The PAS assay consists of two reactions, oxidation of the glycoprotein by periodic acid followed by the condensing of Schiff's reagent (containing colourless Fuchsin-leucosulphonic acid) with the periodate-oxidised glycoprotein (containing aldehyde groups). Sulphonic acid and a glycoprotein-Fuchsin complex is produced which is red in colour. Proteolytic inhibitors could prevent access of the reagents to the sugars and interfere with ring cleavage and aldehyde formation or they could somehow interfere with colour development. Regardless of the mechanisms involved, the presence of proteolytic inhibitors reduced the estimation of glycoprotein content in middle ear effusions by a factor of 5.9 in this assay.

Few studies have been undertaken to analyse individual effusions on the basis of their rheological and biochemical properties and relate effusion rheology to composition (Carrie *et al.*, 1992; Johnson *et al.*, 1997). In this study the viscosity of mucoid and serous effusions (n=50) was measured after mild homogenisation in a phosphate buffer containing proteinase inhibitors. Various research groups have attempted to measure dynamic viscosity of effusions without dilution by subjecting a small volume of effusion (4µl) to cyclic deformation and observing the deformation response of the

effusion (Majima *et al.*, 1988; Takahashi *et al.*, 1990) rather than measuring viscosity over a range of shear rates. It is necessary to measure the viscosity of the effusions over a wide range of shear because the rheological properties of mucin-containing secretions are shear-dependent (Bell *et al.*, 1984) therefore the Contraves low shear viscometer was used to measure effusion viscosity over a shear rate of 0.018s^{-1} to 128.5s^{-1} in the current studies. Measuring only a $4\mu\text{l}$ sample rather than the entire effusion could mean that the rheology of this small amount would not relate to the whole effusion because of non-homogeneity. In the current study, as the whole effusion is measured (after dilution and homogenisation) a more homogeneous and therefore more accurate situation is obtained.

The effusions had been classified by clinical inspection as mucoid or serous and there was a significant difference in viscosity between the two types ($P=0.0061$, unpaired t test). On the basis of rheological properties, the effusions represent two different groups (mucoid and serous) as shown by Carrie *et al.* (Carrie *et al.*, 1992) and it has previously been demonstrated by measurement of endogenous proteinase activity in effusions (using the N-terminal assay at pH7.4) that the difference in viscosity is not due to the level of degradation of the effusion components, but rather due to the relative amounts, composition and structure of the components in the effusions (Carrie *et al.*, 1992) as there was no significant difference in endogenous proteinase activity between the two types of effusion. The lack of mucin degradation in the effusions was confirmed in the present study using SDS PAGE, where mucin remained at the point of application or moved to the interface of the stacking and running gels and there was no evidence of smaller molecular weight glycoprotein.

The NDS content of effusions ranged from 0.49 to 41.3mg per effusion with a mean of 13.29 ± 1.27 (n=50) and there was a significant difference between the content in the two effusion types ($P=0.0381$, unpaired t test) demonstrating that the mucoid effusions contain more material and are more concentrated than the serous effusions, which agrees with previous studies (Carrie *et al.*, 1992; Chung *et al.*, 2002).

Due to the presence of proteinase inhibitors in the extraction buffer, the glycoprotein content was underestimated by approximately 5.9 fold. When the levels were adjusted to account for this the glycoprotein content of the NDS ranged from 0.31 to 13.05mg per effusion with a mean of 3.84 ± 0.44 (n=50). This data can also be expressed as % of glycoprotein per non-dialysable solids and ranged from 11 to 61% of NDS with a mean of 29 ± 1.9 (n=50). As these measurements are just an estimation, they cannot be directly compared to previous data, however they do roughly agree (Fitzgerald *et al.*, 1989; Carrie *et al.*, 1992).

There was a significant correlation between effusion specific viscosity and total NDS and also between specific viscosity and glycoprotein content. Viscosity can be defined as resistance to flow and in polymer-containing material such as effusion homogenates the viscosity will depend on the concentration of the solution (the more concentrated the solution, the more viscous) and the shape and size of the molecules present (small spherical molecules will flow more easily than stiff or extended molecules such as mucins). Glycoprotein has been shown to be the only constituent of effusions that determines the specific viscosity (Fitzgerald *et al.*, 1989; Carrie *et al.*, 1992).

DNA is also a polymeric component of effusions and it is possible that endogenous DNA could have an effect on the viscosity of effusions mediated via an interaction with mucin (Lethem *et al.*, 1990) as it does in respiratory secretions from patients with cystic

fibrosis. In the study by Lethem *et al.* it was shown that 40 μ g of DNA per mg dry weight of mucus gel has a small effect on mucus gel viscosity in cystic fibrosis, 10 and 30% for a chronic bronchitic mucus gel and a fibrotic mucus gel, respectively. However the DNA content of effusion homogenates has been found to be 4.5 μ gmg⁻¹ NDS (Johnson *et al.*, 1997), which is around nine times lower than the levels in the study by Lethem *et al.* and this level is not important in determining the viscosity of the effusions. It has also been shown that after incubation of effusions with DNase for 24 hours, the viscosity of the effusions remained the same (Carrie *et al.*, 1992; Piezhong *et al.*, 2000) whereas proteinase digestion of the effusion homogenate resulted in a large drop in viscosity resulting from degradation of the mucin polymeric structure (Fitzgerald *et al.*, 1989). As there is no significant correlation between protein content and specific viscosity this data demonstrates that the only component of effusions responsible for their viscosity is mucus glycoprotein.

CsCl density gradient centrifugation of each effusion NDS was used to separate the effusion components by their buoyant densities. The fractions with densities of 1.42–1.55gml⁻¹ were pooled for each effusion as these were known to be the glycoprotein-containing fractions (Fitzgerald *et al.*, 1987) and this mucin-rich material was used to determine the mucin gene products present in the effusions and also to look at the degradation state of the mucin.

SDS PAGE was used to demonstrate the degradation state of the mucin from different effusions as this method has previously been validated for measuring the proportions of polymeric and degraded mucin (Rankin *et al.*, 1995). Due to the storage conditions of the effusions before analysis (-20°C) and the presence of proteinase inhibitors before

viscosity measurements were taken, the effusions should have been subjected to minimal *in vitro* degradation.

83.5% \pm 2.4% of the mucin from effusions was of high molecular size and remained at the point of application in the stacking gel of a 4-15% polyacrylamide gel. The remainder of the material was present in the running gel adjacent to the interface of the stacking and running gels. There is little evidence of degradation of the mucin in the effusions and any differences in viscosity cannot be accounted for by changes in polymeric structure of the mucin. The lack of mucin degradation shown in these effusions would mean that they could remain viscous for long periods of time, leading to the long-term lack of mucociliary clearance.

Analysis of the purified mucin from individual effusions was undertaken using the TEPA antiserum (high MUC5B specificity as discussed in Chapter 4) and anti-human gastric mucin antibody NCL-HGM-45M1 (which recognises an epitope in the non-glycosylated part of MUC5AC). Only effusions classified as mucoid were used in this study as they contain more mucin than serous effusions (Fitzgerald *et al.*, 1988). Two effusions were excluded from the study as the levels of MUC5B measured fell out of the normal range and were 6.5 and 20.6-fold higher than the others.

MUC5B was present in all effusions measured in the range 0.006–11.35 μ g per effusion with a mean of 2.13 \pm 0.46 (n=34). MUC5AC was present in 32 of 34 effusions in the range 0.002–1.56 μ g per effusion with a mean of 0.16 \pm 0.06 (n=32). There was approximately thirteen times more MUC5B present compared to MUC5AC in the effusions positive for both with the range (MUC5B:MUC5AC) 1:1 to 160:1.

These results agree with the study by Hutton *et al.* (Hutton *et al.*, 1998a) where mucin from 'thick' and 'thin' effusion pools were analysed for the presence of MUC5B and MUC5AC using relevant antibodies in an ELISA. Only a maximum of 15% of the thick mucin weight was calculated to be MUC5AC and was therefore a minor mucin in the secretions. There was a strong reaction between the mucin and the MUC5B antibody. This antibody reacts well with human salivary mucin, of which MUC5B is the major mucin and studies documented in this thesis have demonstrated that the MUC5B antibody does not react with MUC7 the minor mucin component in saliva. These data suggest that the human middle ear mucosa in glue ear secretes more than one mucin and that MUC5B is a major component whilst MUC5AC is a minor one.

MUC5AC is a normal respiratory epithelium mucin and has been shown to be present in airway secretions pooled from healthy individuals (Thornton *et al.*, 1996). MUC5AC gene expression has not been detected in middle ear mucosa samples from patients without middle ear disease (Lin *et al.*, 2001) using *in situ* hybridisation but mucin gene expression and the translation product have been found in middle ear mucosa from patients with otitis media with effusion (Hutton *et al.*, 1998b; Severn *et al.*, 1999) using immunohistochemistry and *in situ* hybridisation. The middle ear epithelium has been classified as a modified respiratory epithelium and therefore may not secrete MUC5AC in the normal situation. If this is the case then the effusion may have resulted from both an upregulation in gene expression (increased MUC5B production) and an alteration in the pattern of expression (MUC5AC secreted in the inflamed ear but not the normal undiseased ear).

To date there are no studies documented where individual effusions have been analysed for the content of MUC5B and MUC5AC and related to the effusion viscosity.

In this study there was a significant correlation between MUC5B and specific viscosity ($P < 0.0001$, Pearson correlation) but no correlation existed between MUC5AC and specific viscosity ($P = 0.503$, Pearson correlation). This data, as well as the high content of MUC5B in individual effusions suggest that MUC5B is the mucin responsible for effusion viscosity, preventing the mucociliary transport system from clearing the mucus from the middle ear and therefore responsible for the build up of a middle ear effusion. It has been shown that expression of the MUC5B gene in middle ear mucosa epithelium correlates with the expression of inflammatory molecules (Kawano *et al.*, 2000), suggesting that inflammation may initiate and maintain the hypersecretory state of the middle ear mucosa, leading to the chronicity of OME.

MUC5B hypersecretion would appear to be a problem in OME, so the design of specific intervention aimed at the MUC5B secretion should be considered. There are two main ways in which this may be achieved, by the use of mucolytics or antisense deoxynucleotides.

Mucolytic therapy has been used to modify the mucin within the effusion and thereby change the rheological properties allowing removal of the effusion by the mucociliary clearance mechanism from the middle ear cleft. Agents used have been N-acetylcysteine, S-carboxymethylcysteine and Nacystelyn. The therapy has been reported to have had limited success (Taylor and Dareshani, 1975; Ramsden *et al.*, 1977; Hughes, 1984) because if the mucolytic agent reduces the viscosity too much, then the mucociliary transport system cannot clear the effusion as it becomes too thin and it was reported from *in vitro* studies that after a 24 hour period, the viscosity of the

effusion increases due to denaturation and aggregation of protein components (Pearson *et al.*, 1997). The concentration of the mucolytic reaching the middle ear and the time it will remain is not known yet is important as some mucolytic agents are cytotoxic over certain concentrations (Pearson *et al.*, 1997).

Antisense deoxynucleotides are sections of DNA, 12 to 30 nucleotides in length that interfere in a sequence specific manner with processes such as the translation of mRNA into protein. They are made to identify a particular target, such as a mucin gene and can block expression of the specific gene within the cell by binding the complementary mRNA sequence, thereby preventing the translation of mRNA. They must be stable *in vivo* and also must be able to enter the target cell and be retained there (Stein and Cheng, 1993). They are currently under investigation in a number of different fields including oncology, cardiovascular diseases and infectious diseases (Agrawal and Iyer, 1995; Wagner and Flanagan, 1997). They would only be of use in OME if they could target the middle ear in particular and would reduce the level of mucus secretion but not remove it altogether as it plays a vital part in the mucociliary escalator. If the antisense deoxynucleotides had no specific target organ they could reduce secretion of the particular mucin, such as MUC5B throughout the body. MUC5B is also secreted in saliva, in the lungs and in bile and reduction in mucin secretion in these areas could have serious consequences.

A subset of effusions (n=14, mucoid) for which MUC5B and MUC5AC content was known, were also analysed for the presence of the cytokines IL-6 and IL-8. Cytokine levels and protein content were measured in the effusion homogenate immediately after viscosity measurements were taken. All data were expressed per mg NDS to account for any changes in hydration that may have occurred during storage of the effusions.

The protein content of effusions varied between 649-961 μgmg^{-1} NDS. These values are very high in comparison with levels previously measured in effusions of 1-266 μgmg^{-1} NDS (Johnson *et al.*, 1997) and 57-230 μgmg^{-1} NDS (Carrie *et al.*, 1992).

This could be due to two reasons:

1. The above studies measured protein content in NDS, whereas in the current study the protein was measured in the entire homogenate therefore it is possible that components were present in the homogenates that could interfere in the assay which were removed in the dialysis stage in the previous studies.
2. In the current study, dilutions of up to four hundred fold of the homogenates were made which were necessary to obtain results relative to the standard curve in the protein assay. This could also lead to inaccuracies in the measurement of total protein content.

The levels of cytokines IL-6 and IL-8 were measured in effusions. They are present in high concentrations in effusions and are known to be the secondary cytokines activated in the cascade by TNF- α and IL-1 β (Maxwell *et al.*, 1994; Sato *et al.*, 1999; Skotnicka and Hassman, 2000). They have been shown using *in vitro* studies to induce mucin secretion (MUC5AC and MUC5B) at different stages and to different levels after

stimulation of a mucus-producing colon carcinoma cell line (HT29-MTX) (Smirnova *et al.*, 2001; Smirnova *et al.*, 2002a).

IL-6 content in 14 effusions ranged from 1.5–558.5pgmg⁻¹ NDS with a mean of 178 ± 42.5 (n=14) NDS and IL-8 content in the same effusions ranged from 61.6-17130pgmg⁻¹ NDS with a mean of 4020 ± 1317 (n=14). There is generally a much greater amount of IL-8 in the effusions than IL-6. Previous studies have expressed cytokine levels per mg protein in the effusions and therefore to directly compare the results from this study with others the data has also been expressed in this way. IL-6 content had a mean of 224.9pgmg⁻¹ protein and the mean value for IL-8 was 5129pgmg⁻¹ protein. Previous studies have shown the mean IL-6 content in effusions to be 173.9pgmg⁻¹ protein (Yellon *et al.*, 1992) and the mean IL-8 content to be 4805pgmg⁻¹ protein (Maxwell *et al.*, 1994), which are levels consistent with those found in the current study. Other studies have found lower levels of IL-8 ranging from 559 to 617pgmg⁻¹ protein (Hotomi *et al.*, 1994; Johnson *et al.*, 1997; Pospiech *et al.*, 2000), which is around eight-fold lower than the current study. The effusion reflects the contents present at effusion removal and the stage of the inflammatory cascade at this point is unknown therefore it is understandable that the cytokine levels will vary between samples. It is also possible that the effusion could be protecting cytokines from degradation and this could explain the very high cytokine levels in a subset of effusions (for example in effusions 6, 8 and 14, section 5.3.4). Retention of cytokines in the middle ear cleft may result in an ongoing inflammatory state with the cytokines continuing to stimulate cells and their products in the middle ear cleft.

There was a significant correlation (P=0.0149) between levels of IL-6 and IL-8 in effusions and this has previously been demonstrated in a chinchilla otitis media model

after inoculation by *Streptococcus pneumoniae* (Sato *et al.*, 1999). It is possible that there is a correlation between the cytokine levels as they are both secondary cytokines in the inflammatory cascade and may be stimulated at similar times by the primary cytokines IL-1 β and TNF- α , or it may be that one cytokine could stimulate the secretion of the other. It has been shown *in vitro* that inflammatory cells such as TNF- α and IL-1 β can stimulate mucosal cells to secrete cytokines (Smirnova *et al.*, 2002a).

IL-6 and IL-8 levels were plotted against MUC5B and MUC5AC content and effusion viscosity and the statistical correlations were determined. There was a significant correlation between reduced specific viscosity (specific viscosity mg⁻¹ NDS) and IL-8 content pgmg⁻¹ NDS (P=0.0267) and also MUC5AC content and IL-8 content pgmg⁻¹ NDS (P=0.004). Both of these correlations were significant only when one data point was excluded. There were no other significant correlations between mucin gene products and cytokine levels. This data agrees with the *in vitro* studies by Smirnova and co-workers (Smirnova *et al.*, 2001; Smirnova *et al.*, 2002a) in that the induction of MUC5AC was much greater than that of MUC5B in the presence of IL-6 and IL-8. The *in vitro* data suggests that IL-6 and IL-8 may have different roles in the middle ear. IL-6 is produced at a lower level than IL-8 in the middle ear and may be responsible for the maintenance of inflammation and prolonging mucin secretion. IL-8 may contribute to the maintenance of chronic OME by inducing a prolonged mucin secretion, which is delayed compared to the induction by other cytokines.

In middle ear effusions there is no knowledge of the stage of disease progression as the effusion is analysed on removal and it is not possible to investigate if the *in vivo* situation mirrors the *in vitro* experiments.

As the absolute amount of IL-8 correlates with the reduced specific viscosity of the effusions and with levels of MUC5AC it may be that MUC5AC is produced in response to IL-8 in the middle ear and that another stimulus or other cytokines are responsible for the upregulation of MUC5B. The reason for the correlation between IL-8 and MUC5AC could be that in the normal uninflamed ear it has been reported that MUC5AC is absent (Lin *et al.*, 2001) and that in the diseased state MUC5AC secretion increases with the presence of IL-8. MUC5B however is expressed at a low level in the normal ear and in the diseased ear the effect of IL-8 on MUC5B is smaller and starts at a much higher level and this starting level masks the effect of IL-8.

Further investigations should focus on the measurement of all cytokine levels in effusions and relating these to the MUC5B and MUC5AC levels. If a correlation between MUC5B, the major mucin, and a particular cytokine is significant then it may be possible to target this cytokine and reduce the induction of MUC5B gene expression.

CHAPTER 6

GENERAL DISCUSSION

Chapter 6 General Discussion

The middle ear is an air-filled chamber containing the auditory ossicles, connected to the nasopharynx by the Eustachian tube. The middle ear secretes mucus, which provides a protective barrier against invading particles and microorganisms and dehydration. The mucus is an integral part of the mucociliary escalator, which transports trapped particles towards the nasopharynx via ciliary beating. Mucins have been shown to be solely responsible for the viscoelastic properties of mucus (Fitzgerald *et al.*, 1989; Carrie *et al.*, 1992) therefore any change in the quantity or type of mucin secreted could alter the clearance of mucus from the middle ear and result in a pathological condition.

Otitis media with effusion (OME) is defined as inflammation of the middle ear with the accumulation of a viscous mucin-rich effusion in the middle ear cleft without signs or symptoms of active infection (Bluestone, 1999). In the disease, inflammation is the initiator of a hypersecretory state that occurs as a result of mucosal differentiation. An increase in the number of goblet cells in the epithelium and submucosal glands is observed resulting in hypersecretion of mucus. The increase in the number of secretory cells parallels a decrease in the number of ciliated cells (Inagaki *et al.*, 1988) and the effusion that accumulates is unable to be cleared and remains in the middle ear cleft. The effusion dampens mobility of the auditory ossicles and impedes sound transmission, resulting in hearing loss whilst the effusion is present.

Due to lack of availability of normal middle ear mucosa, there is little data regarding the mucin genes expressed in the undiseased state. Lin *et al.* (Lin *et al.*, 2001) reported the expression of the MUC5B gene and the presence of the gene product in normal middle ear mucosa, however gene products present in the normal mucus secretion were not

measured. In middle ear mucosa samples from children with OME, expression of MUC1, MUC2, MUC5AC, MUC5B and MUC7 was detected using *in situ* hybridisation, the highest intensity of expression was observed with the secreted mucins MUC5B in submucosal glands and MUC5AC in goblet cells and the membrane bound mucin MUC4 present at the surface epithelium (Severn *et al.*, 1999; Kawano *et al.*, 2000). Using immunohistochemical analysis, MUC5AC and MUC5B gene products were detected in middle ear mucosa (Hutton *et al.*, 1998b) with the same cell distribution as observed using *in situ* hybridisation, demonstrating that gene expression is related to cell type. Tissue expression does not always correlate with the gene product in the secretion (Thornton *et al.*, 1996) therefore it was necessary to analyse the mucin gene products present in effusions. Previous studies using pooled effusions have shown that MUC5B and MUC5AC are present (Hutton *et al.*, 1998a) however the work presented in this thesis is the first study using individual effusions.

It was demonstrated using dilute solution viscosity studies that the specific viscosity of effusions significantly correlated with the total glycoprotein content, supporting the theory that mucins are responsible for the rheological properties of the effusion (Chapter 5). The mucins present were measured following purification of effusion glycoprotein by CsCl equilibrium density gradient centrifugation. MUC5B was present in all effusions and MUC5AC was found in 94% of these. MUC5B content was on average thirteen times the level of MUC5AC demonstrating that MUC5B is the major mucin present in effusions with MUC5AC secreted at much lower levels. There was a significant correlation between MUC5B content and effusion specific viscosity. From the results presented in this thesis one can conclude that in OME there is an upregulation of MUC5B, the mucin secreted in the normal middle ear and also an

alteration in the pattern of mucin expression as MUC5AC can be detected only in the diseased state, however this has only been investigated in one study. Middle ear epithelium is modified respiratory in type and MUC5AC is a major secretion in the airways (Gendler and Spicer, 1995). It would be interesting to further this study by identifying the mucin genes expressed and secreted in the normal middle ear to clarify that MUC5AC is not secreted in the undiseased state, however middle ear mucosa specimens are generally unavailable.

Middle ear mucin remains largely undegraded as demonstrated by SDS PAGE, this lack of degradation would mean that the effusions could remain viscous for long periods and be immovable by the cilia and therefore may be present in the middle ear for longer than three months.

In order to quantify the MUC5B mucin present in effusions it was necessary to prepare a MUC5B standard, as there was no appropriate standard available. This was achieved by CsCl equilibrium density centrifugation of human saliva. There are two main secreted mucin populations present in human saliva, MUC5B and MUC7 (Prakobphol *et al.*, 1982). There is documented evidence of MUC4, a membrane bound mucin in the submandibular and sublingual glands (Troxler *et al.*, 1997) measured by Northern blotting and it is possible that this mucin is also present as a minor component in the saliva secretion if it is in a soluble form or as a cleavage product. The CsCl purified saliva was highly reactive against the MUC5B antisera (TEPA) and was not reactive with the MUC7 antisera suggesting that the purified mucin consisted of mainly the MUC5B gene product and was a relatively pure standard to use in the quantification of MUC5B.

OME is an inflammatory disease and many inflammatory mediators have been identified in the effusion including arachidonic acid metabolites, histamine, immunoglobulins, components of the complement system and cytokines. Cytokines are a group of low molecular weight glycoproteins of which IL-1 β , IL-2, IL-6, IL-8, IFN γ and TNF α have been previously measured in effusions (Yellon *et al.*, 1991; Himi *et al.*, 1992; Maxwell *et al.*, 1994; Takeuchi *et al.*, 1994; Yellon *et al.*, 1995; Johnson *et al.*, 1997; Pospiech *et al.*, 2000; Schousboe *et al.*, 2001). *In vitro* studies using a human colon carcinoma-derived mucus secreting cell line (HT29-MTX) have shown that IL-6 and IL-8 have the ability to stimulate MUC5AC and MUC5B secretion at different rates, for different periods of time and to different levels (Smirnova *et al.*, 2001; Smirnova *et al.*, 2002a; Smirnova *et al.*, 2002b) and that they may have roles in different stages of the inflammatory process. In the current study, IL-6 and IL-8 levels were measured in effusions and compared to levels of MUC5B and MUC5AC. A significant correlation was observed between the IL-6 and IL-8 content of effusions suggesting that the cytokines are either stimulated at similar times or that one cytokine may stimulate the secretion of the other. The IL-8 content correlated significantly with MUC5AC in effusions, this agrees with the above *in vitro* results of Smirnova *et al.*, and no other significant correlations were observed between IL-6, IL-8, MUC5B and MUC5AC. It may be that MUC5AC is produced in response to IL-8 in the middle ear and that another stimulus or other cytokines are responsible for the upregulation of MUC5B. It is not possible to conclude if the *in vitro* situation mirrors the *in vivo* situation from this study as the stage of the disease and therefore the inflammatory cascade is unknown at the time of effusion removal.

To further this work, levels of all cytokines could be measured in effusions and related to the presence of MUC5B and MUC5AC to investigate if any other cytokines correlate with MUC5AC secretion and to identify if any cytokines are linked to secretion of MUC5B, which is the major mucin. It would be useful to collect data on the time of initial diagnosis to have an idea of duration of the disease (and stage of the inflammatory process) in each individual.

Mucolytic therapy has been used to treat OME by altering mucin structure within the effusion and thereby reducing effusion viscosity. This treatment had only limited success (Taylor and Dareshani, 1975; Ramsden *et al.*, 1977; Hughes, 1984) as there is an optimum viscosity at which mucus is effectively transported (Silberberg, 1983) and if the effect of the mucolytic resulted in the effusion becoming too thin or remaining too viscous then it could not be removed by the cilia from the middle ear cleft. Currently OME is treated by surgical removal of the effusion, a process termed myringotomy, and insertion of a grommet into the tympanic membrane to allow aeration of the middle ear. This procedure treats the symptoms of OME rather than the cause. Retention of cytokines in the middle ear cleft may result in an ongoing inflammatory state with the cytokines continuing to stimulate cells and their products in the middle ear cleft, especially if the effusion is protecting them from degradation. It may be possible to target and deactivate these inflammatory mediators in the middle ear by direct injection through the tympanic membrane. This would reduce the inflammatory process in the middle ear cleft and prevent the chronic stages of OME, especially if the stimulus for MUC5B secretion could be identified.

There are many inflammatory stimuli associated with OME such as bacteria and their products, viruses and allergy (Giebink, 1989; Arola *et al.*, 1990; Mogi *et al.*, 1992; Post

et al., 1995; Shaw *et al.*, 1995; Liederman *et al.*, 1998; Post *et al.*, 1998). A link between OME and gastro-oesophageal reflux has also been suggested by some researchers (Shilkin, 1994; Halstead, 1999; Velepich *et al.*, 2000) however a direct relationship has not until now been determined. In the current study, acidic proteinase activity was detected in 29% of effusions and pepsin/pepsinogen protein levels were up to 1000 times higher than serum reference levels. Albumin and fibrinogen levels in effusions were of the same order as serum reference levels indicating that serum proteins were not being concentrated up in the effusions. Preliminary immunohistochemical analysis of middle ear mucosa biopsies demonstrated that the middle ear does not secrete pepsin therefore the only likely source of pepsin is from reflux of gastric juice.

The presence of acidic reflux in the nasopharynx has previously been documented in a 24 hour pH monitoring study (Contencin and Narcy, 1991) and reflux of gastric contents from the nasopharynx to the middle ear is possible in infants and young children due to anatomic differences in the Eustachian tube in this age group compared to children aged seven and above (Bluestone, 1996). The Eustachian tube is shorter and has a lower angle of inclination in infants and young children (Sadler-Kimes *et al.*, 1989) thus allowing gastric contents to reach the middle ear more easily and the tensor veli palatini muscle, which is involved in the active opening and closing mechanism of the Eustachian tube is less efficient enabling unwanted secretions such as reflux or those containing bacteria to gain access to the middle ear. Gastric contents are acidic, (however due to neutralising bicarbonate in the oesophagus, they would not be as acidic as in the stomach) and would cause damage and inflammation in the Eustachian tube and middle ear leading to the accumulation of an effusion, which could block the

immature Eustachian tube due to the lumen being narrow. Pepsin would be inactive due to the high pH of the effusion. If the Eustachian tube remains unblocked and a second reflux event occurs, pepsin present from the previous reflux episode may be reactivated as the work presented in this thesis has demonstrated that effusion contents protect pepsin from complete denaturation at up to pH8 and further damage and inflammation would result. Due to the damaged mucosal epithelium, bacteria present in the middle ear could easily invade the tissue, cause a secondary infection and prolong the inflammation, leading to a chronic disease state. In conclusion, gastro-oesophageal reflux may be the primary factor in the initiation of OME in children as a decrease in the prevalence of OME correlates with the anatomical changes that occur upon maturity including a change in the angle of the Eustachian tube to 45° to the horizontal, an increase in the tube size and mechanical efficiency for tubal opening (Sadler-Kimes *et al.*, 1989). Anti-reflux therapy could be particularly relevant in the group of children who require repeated grommet insertion or have therapy resistant middle ear disease as they may be suffering from OME induced by persistent gastric reflux and anti-reflux therapy would potentially cure the disease.

To further this work, effusions could be analysed for other components of gastric juice such as gastric lipase or intrinsic factor to further confirm the role of reflux in OME. Few long-term studies on OME have taken place due to the lack of a suitable and reliable model. It may be possible to set up an animal model for reflux by instilling gastric juice into the middle ear via the Eustachian tube and observe whether an effusion develops rather than as Heavner *et al.* (Heavner *et al.*, 2001a; Heavner *et al.*, 2001b) where a rat model was exposed to gastric juice components via the tympanic membrane, which is non-physiological.

The overall conclusions from this PhD study are that OME is an inflammatory disease resulting in hypersecretion of mucus. The effusion is viscous and has high mucin content, most of which consists of MUC5B however MUC5AC is also present as a minor mucin. Gastro-oesophageal reflux may be the primary cause of OME, instigating a cascade of inflammatory events leading to the conditions seen in the disease.

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